Volume 18 1952

ANTONIE VAN LEEUWENHOEK

Journal of Microbiology and Serology

Official publication of the
„NEDERLANDSCHE VEREENIGING VOOR MICROBIOLOGIE"
(Netherlands Society of Microbiology)

Board of Editors:
A. CLARENBURG, Utrecht; H. W. JULIUS, Utrecht;
A. J. KLUYVER, Delft; A. CHARL. RUYS, Amsterdam;
JAN SMIT, Wageningen; E. P. SNIJDERS, Amsterdam;
J. D. VERLINDE, Leiden; JOHA. WESTERDIJK, Baarn.

Editors:
MARIE P. LÖHNIS, Wageningen
L. H. C. PERQUIN, Breda

SWETS EN ZEITLINGER
BOEKHANDEL EN UITGEVERSMAATSCHAPPIJ — AMSTERDAM

CONTENTS TABLE DES MATIÈRES INHALT

Page

ALLEN, O. N. s. JOHNSON, M. D.

VON ARX, J. A., Ueber die Ascomycetengattungen *Ceratostomella* Sacc., *Ophiostoma* Syd. und *Rostrella* Zimmermann 201

BANNINK, H. F. and MULLER, F. M., On the utilization of waste liquors from the digestion of straw with monosulphite 45

BEEM, B. s. VERLINDE, J. D.

BOOY, HANS L. and WOLFF, HENDRIK L., On the induction of Vi-antigen formation in a strain of *Salmonella typhi* free of Vi-antigen 183

BRANDWIJK, A. C. s. TASMAN, A.

BRANS, L. M. s. MULDER, J.

BUSNEL, M. C. s. VAGO, C.

BIJLMER, J., The susceptibility of *Spirillum minus* and *Borrelia duttoni* to streptomycin and penicillin 246

CLARENBURG, A., HEMMES, G. D. and WAGENVOORT, W., On the occurrence of *Salmonella bareilly* in man and animals and the mode of transmission of the salmonellose caused by this species 171

COLLIER, W. A. und WINCKEL, W. E. F., Beiträge zur geographischen Pathologie von Suriname. 6. Histoplasmose bei Säugetieren in Suriname 349

DEKKING, F., A cheap and safe method of keeping mice 182

——, Nicolau stain for smallpox diagnosis 291

DEVOS, A. s. GEURDEN, L. M. G.

EMMERIE, A. s. KLIP, W.

ENGEL, CHR. s. KLIP, W.

GEST, H. and STOKES, J. L., The effect of carbon dioxide on reduction of methylene blue by micro-organisms 55

GEURDEN, L. M. G. and DEVOS, A., Studies of the antigenic properties of fowl erythrocytes 215

GISPEN, R., Silver impregnation of smallpox elementary bodies after treatment with xylol 107

GREMMEN, J., A preliminary study of the culture of *Discomycetes*, especially the perfect stage 153

HEMMES, G. D. s. CLARENBURG, A.

HERS, J. F. PH. s. MULDER, J.

KAARS SIJPESTEIJN, A. and VAN DER KERK, G. J. M., Investigation on organic fungicides. VI. Histidine as an antagonist of tetramethylthiuram disulphide (T.M.T.D.) and related compounds 83

64-00529

Per.
QR
1
A58
v. 18

Page

van der Kerk, G. J. M. s. Kaars Sijpesteijn, A.

Klarenbeek, A. s. Verlinde, J.

Klip, W., The tuberculostatic action of vitamin D_2 217

——, Emmerie, A. and Engel, Chr., The tuberculostatic properties of the unsaponifiable fraction of cod-liver oil 227

Kluyver, A. J., In Memoriam H. C. Jacobsen 167

Kneteman, A., Enrichment and isolation of *Streptococcus citrophilus* van Beynum et Pette 275

Johnson, M. D. and Allen, O. N., Cultural reactions of rhizobia with special reference to strains isolated from *Sesbania* species 1

—— and ——, Nodulation studies with special reference to strains isolated from *Sesbania* species 13

Lukes, T. M. and Phaff, H. J., Characteristics of trehalase in *Candida tropicalis* 323

Marseille, A. s. Tasman, A.

Mulder, J., de Nooyer, I. and Brans, J. M., The influence of treating ferret influenza antisera with enzymes of crude filtrate of *Vibrio cholerae* on the titre of the antibodies 128

——, Brans, L. M. and Hers, J. F. Ph., Studies on the increase of antibodies in nasal secretions of human beings after intracutaneous vaccination with influenza virus, with the aid of the haemagglutination inhibition technique 131

—— and Brans, L. M., Studies on the antigenic behaviour of egg- and egg-mouse-egg-lines of strains of influenza virus, with the aid of the haemagglutination-inhibition test 139

Muller, F. M. s. Bannink, H. F.

de Nooyer, I. s. Mulder, J.

Oskam, H. J., Comparison of two methods for the determination of the resistance of *Mycobacterium tuberculosis* to p-aminosalicylic acid and streptomycin 316

Phaff, H. J. s. Lukes, T. M.

Pijper, A. s. Spruit, C. J. P.

van Ramshorst, J. D. s. Tasman, A.

Richard, O. s. Wikén, T.

Scholtens, R. Th., The formation of bacteriophages in mixed cultures by recombination of genetic elements; characterisation of phage types of *Salmonella paratyphi* B by phage reactions and lysogenic properties 257

van der Slot, J. s. Tasman, A.

Spruit, C. J. P. and Pijper, A., An obligate halophilic bacterium from solar salt 190

Page

Stokes, J. L., Inhibition of microbial oxidation, assimilation and adaptive enzyme formation by methylene blue 63
——, s. Gest, H.

Tasman, A., Brandwijk, A. C., Marseille, A., van Ramshorst, J. D. and van der Slot, J., On the purification and adsorption of staphylococcus toxoid 336

—— and van Ramshorst, J. D., On the preparation and properties of a new tetanus-prophylactic, the ,,Tetanus-P.T.'' 357

van Tongeren, H. A. E. s. Verlinde, J. D.

Vagò, C. et Busnel, M. C., Photosensibilité du *Bacillus cereus* var. *Alesti* au rayonnement ultraviolet de 2537 Å 125

Verlinde, J. D. and van Tongeren, H. A. E., Isolation of smallpox virus from the nasopharynx of patients with variola sine eruptione . 109

—— and ——, A mixed epidemic of poliomyelitis and Bornholm's disease (pleurodynia) 239

—— and Beem, B., Haematogenous spread of poliomyelitis virus . . . 251

——, Beem, B. and Klarenbeek, A., Studies on immunity in poliomyelitis. II. Adaptation of a strain of poliomyelitis virus on mice, and immunization experiments with the mouse-adapted virus 364

Wagenvoort, W. s. Clarenburg, A.

Welsch, Maurice, Streptomycine et lysozyme 23

Wikén, T. und Richard, O., Untersuchungen über die Physiologie der Weinhefen. II. Mitteilung. Wachstumsbedingungen weiterer auxo-autotropher, schweizerischen Kulturweinhefen 31

—— und ——, Untersuchungen über die Physiologie der Weinhefen. III. Mitteilung. Ueber den Wuchsstoffbedarf einer auxo-heterotrophen, schweizerischen Kulturweinhefe 293

Winckel, W. E. F. s. Collier, A.

Wolff, Hendrik L., Experiments with an apparatus for lyophile desiccation of micro-organisms 113

——, s. Booy, Hans. L.

Printed in the Netherlands by Koch & Knuttel Ltd., Gouda

(From the Department of Bacteriology, University of Wisconsin, Madison 6, Wisconsin).

CULTURAL REACTIONS OF RHIZOBIA WITH SPECIAL REFERENCE TO STRAINS ISOLATED FROM *SESBANIA* SPECIES [1]

by

M. D. JOHNSON and **O. N. ALLEN** [2]

(Received July 31, 1951).

This investigation was undertaken with the objective of augmenting our knowledge about the cultural characteristics of the rhizobia capable of producing nodules on species of the genus *Sesbania*. In general, the majority of species in this genus abounds in semitropical and temperate areas. Because of the rapid growth and heavy tonnage produced, *S. aculeata* Poir., *S. longifolia* DC. and *S. macrocarpa* Muhl. and Rafin. are popular as green manures and in soil conservation practices. *S. macrocarpa* is best known as a shade crop in irrigated areas. According to a Japanese patent MATSUOKA (13) has described a process by which stems of *S. aegyptiaca* Poir., after special chemical treatment, can be used in a manner similar to hemp fiber. Leaves of *S. grandiflora* Poir. are reportedly an excellent source of ascorbic acid (5).

In general, species of this genus are abundantly nodulated, yet little knowledge exists as to the descriptive character of the rhizobia involved. The earliest descriptions of sesbania rhizobia were given by RAJU (15), who observed that 5 strains from dhaincha (*S. aculeata*) exhibited variations in colonial size and appearance on yeast water mannitol agar and in the production of serum zones in litmus milk. The comprehensive study by HARRIS (7) included a greater number of strains and a wider diversity of parent host plants although the scope of his studies did not allow for a differentiation of these bacteria from other rhizobia.

---

[1] Published with the approval of the director, Wisconsin Agricultural Experiment Station.

[2] Research assistant and Professor of Bacteriology, respectively.

This investigation explores comparative cultural and physiological reactions of 39 strains of rhizobia from 6 species of the genus *Sesbania*, 52 strains of rhizobia from plants contained in 7 of the more common cross-inoculation groups, 8 strains of *Agrobacterium radiobacter* and 10 strains of *A. tumefaciens*. Also included is an appraisal of the accuracy and value of certain differential tests for the separation of rhizobia from the closely related species in the genus *Agrobacterium*.

METHODS.

The sources of the various cultures reviewed in this study are shown in Table 1.

The majority of the media used in this study was sterilized in the autoclave at 15 pounds pressure for 45 minutes. Intermittent sterilization (10 pounds pressure for 15 minutes on 3 consecutive days) was employed when the media were of such a chemical nature that high temperature sterilization was not advisable. Media which could not be heated as described above were sterilized by filtration through a Seitz filter. Inoculation of all media was made with a standard loop (0.04 ml) of a turbid culture of the organisms cultivated in yeast water mannitol broth, better known as medium 79 (3). All cultures were incubated at room temperature (25—28° C.) and examined at periodic intervals over a period of 3 weeks.

The reactions in veal infusion broth were recorded after 5 days incubation, in HOFER's yeast extract mannitol broth (pH 10) at 6 days and in the same medium at pH 11 at 12 days. The medium of RIKER *et al.* (17) used in the nitrate reduction tests was modified by the substitution of glucose for sucrose and the addition of 8 ml yeast water per liter.

For the sake of brevity reference citations are given for the composition of the media instead of enumerating the detailed formulae.

RESULTS AND DISCUSSION.

Prior to the cultural tests the strains were screened for purity by repeated streaking and the isolation of well separated colonies from yeast water mannitol agar plates. Many of the strains were progenies of recent single cell isolations. The abilities of the many rhizobial strains to form nodules on their proper host plants were confirmed in earlier experiments; similarly, other tests showed that the 10

TABLE 1.

Sources of sesbania rhizobia and related organisms.
(*Host plants and strain numbers)

Organisms Studied

- *Rhizobium* sp. (?)
  - **S. macrocarpa* Muhl. and Rafin. – 762, 763, 764, 765, 773, 774, 775, 1050
  - *S. longifolia* DC. – 776, 777, 1051, 1052, S-1 to S-10, incl.
  - *S. grandiflora* Poir. – 780, 781, 782, 783, 784, 785, 788
  - *S. aculeata* Poir. – 789, 791
  - *S. sesban* (Linn.) Merr. – 792, 793, 794
  - *S. tomentosa* Hook. and Arn. – 768, 769, 771, 772, 786

- *Rhizobium meliloti*
  - *Medicago sativa* L. – 100, 101, 121
  - *Medicago lupulina* L. – 120
  - *Melilotus alba* Desv. – 114, 122, 123
  - *Trigonella foenum-graecum* L. – 137

- *Rhizobium trifolii*
  - *Trifolium pratense* L. – 201, 203, 204, 207, 208, 210
  - *Trifolium incarnatum* L. – 209
  - *Trifolium procumbens* L. – 214

- *Rhizobium leguminosarum*
  - *Pisum sativum* L. – 314, 315
  - *Pisum sativum* L. var. *arvense* Poir. – 320, 321, 322, 323, 326
  - *Vicia angustifolia* (L.) Reichard – 331

- *Rhizobium phaseoli*
  - *Phaseolus vulgaris* L. – 403, 405, 409, 412

- *Rhizobium japonicum*
  - *Glycine max* (L.) Merr. – 502, 505, 506, 508, 513, 517, 518, 526.

- Cowpea rhizobia
  - *Vigna sinensis* Endl. – 600, 601, 602, 610, 611
  - *Phaseolus limensis* Macf. – 603
  - *Indigofera suffruticosa* Mill. – 612
  - *Laburnum vulgare* L. – 613

- *Rhizobium lupini*
  - *Lupinus* sp. – 801, 802
  - *Lupinus arboreus* Sims. – 803
  - *Lupinus nanus* Douglas – 804
  - *Lupinus albus* L. – 806, 807
  - *Lupinus mutabilis* Sweet – 810
  - *Ornithopus sativus* Brot. – 814

- *Agrobacterium radiobacter* – 1000, 1001, 1003, 1005, 1007, 1008, T-1, 120

- *Agrobacterium tumefaciens*
  - *Phaseolus* sp. (bean) – T–2
  - *Malus* sp. (apple) – T–3
  - *Juglans* sp. (walnut) – T–4
  - *Syringa* sp. (lilac) – T–5
  - *Chamaecyparis* sp. (white cedar) – T–7
  - *Bellis* sp. (daisy) – T–8
  - *Olea* sp. (olive) – T–9
  - *Lycopersicon* sp. (tomato) – T–10
  - *Amygdalus* sp. (peach) – T–11
  - *Humulus* sp. (hop) – T–12

strains of *A. tumefaciens* possessed the ability to incite gall formation on tomato plants.

1. Sesbania Rhizobia.

All the sesbania isolates were typical rhizobia as evidenced by their being Gram-negative short rods (1.0—2.0 μ in length) and slightly swollen in the bacteroidal stage. Young cells showed considerable affinity for stains. Older cultures exhibited variations in cell shapes, sizes and the usual irregular staining characteristic of rhizobia.

The rates of growth of the sesbania strains varied within narrow limits. Two types of colonies on all agar media were soon recognized: those organisms isolated from nodules of *S. macrocarpa* and *S. longifolia* produced large, moist, raised, mucilaginous colonies; those from *S. grandiflora, S. tomentosa, S. aculeata* and *S. sesban* were smaller in diameter, more compact, opaque and less mucilaginous. Further differences between these two groups are shown later in this report.

All the sesbania strains gave alkaline reactions with pronounced serum zones in litmus milk. The following plant extract mineral salts media containing 1 per cent mannitol as the carbon source are arranged in the following ascending order relative to affording abundance of growth: 10 per cent kraut juice, Albrecht and McCalla's calcium gluconate sauerkraut medium (1), 10 per cent asparagus extract (3), yeast extract (3) and 5 per cent yeast plus 5 per cent potato extracts.

Carbohydrate utilization by the sesbania strains was ascertained by adding 1 per cent of the respective carbon source to yeast water mineral salts agar containing 5 ml of a 0.4 per cent solution of brom thymol blue per liter. All the strains produced slightly acid reactions when 1 per cent arabinose, rhamnose, galactose, lactose, glucose and erythritol were employed; slightly alkaline reactions were obtained with sucrose, mannitol and dulcitol. No changes in reaction were noted with levulose and maltose. With the exceptions of the last 5 sugars, the reactions of these strains markedly resembled the reactions produced by the fast-growing *Rhizobium* spp., typified by the test strains of *R. meliloti, R. trifolii, R. leguminosarum* and *R. phaseoli*. Alkaline reactions were produced by the test strains of *R. japonicum, R. lupini* and cowpea rhizobia in the presence of all carbon sources. Abundant growth accompanied by an acid reaction was produced by the sesbania strains in the presence of 0.5 per cent of the sodium salts of malic, citric, formic, lactic, succinic, tartaric and acetic acids in respective media. Much less growth was produced in the mineral salts medium to which 0.5 per cent mannitol was added as a carbon source and when 1 per cent potassium nitrate, peptone, ammonium sulfate, respectively, were used as a source of nitrogen. None of the strains was able to utilize nitrite nitrogen. All strains produced a positive tyrosinase reaction.

2. COMPARATIVE TESTS.

A summarization of additional comparative tests between the sesbania rhizobia and other diverse strains is contained in Table 2. Of primary importance here are the physiological differences between the two groups of sesbania rhizobia, yet all these isolates showed many cultural similarities with the other rhizobia tested. The media of GEORGI and ETTINGER (6), in addition to certain others listed, were particularly satisfactory in differentiating between the rhizobial strains and related ones in the genus *Agrobacterium*. Diverse cultural differences between members of the genera *Rhizobium* and *Agrobacterium* as proposed by other workers have been summarized recently by ALLEN and ALLEN (2).

Forty-two of the 91 strains of rhizobia studied showed dye-absorbing colonies on Congo red agar (3, 4). Notably these variants were not among the slow-growing strains of *R. japonicum*, *R. lupini* and cowpea rhizobia. Subsequent to meticulous isolation and purification, these variants gave identical reactions with those produced by the non-dye-absorbing colonies of the same strain. Because of this and the fact that all of them produced nodules on their host plant, none incited gall formation on tomato plants, and none simulated strains of *A. radiobacter*, it is fitting to conclude that these variants were typical rhizobia. An explanation for this variation is not available at the present time. The data reported here concern only the reactions produced by the rhizobial isolates whose colonies were glistening, translucent and persistently white on this medium. The strains of *A. tumefaciens* and *A. radiobacter* produced brilliantly red colonies. In the light of this aforementioned variation, question is raised here that this medium is a reliable criterion in the recognition of rhizobial strains as determined solely by dye absorption.

Rather interesting variations were obtained in the tolerance of

TABL

Comparative studies of rhizobia

Media	Sesbania rhizobia		*R. meliloti*	*R. trifolii*
	Group 1*	Group 2**		
Number strains tested:	22	17	8	8
Litmus milk	Alk s.z.	Alk s.z.	A s.z.	A s.z.
Rapid nitrate reduction (17)	—	—	—	—
Slow nitrate reduction	—	+	—	—
Growth in:				
Hofer's medium, pH 10. (9, 10)	0	+	+	0
Hofer's medium, pH 11. (9, 10)	0	0	0	0
Veal infusion broth (8, 10)	0	+	+	0
Uric acid broth (10, 12)	0	++	0	0
Formate ricinoleate medium (10)	0	0	0	0
Clark and Lub's medium (2, 10)	0	+	0	0
ZoBell's medium (20)	0	+	0	0
H_2S production, Zo Bell's medium	0	0	0	0
Browning of calcium glycero-phosphate medium (16)	—	—	—	—
Yeast extract, mineral salts medium + $CaCO_3$ +, (6)				
1 % dextrin	Alk	Alk	Alk	Alk
1 % inulin	Alk	Alk	Alk	Alk
1 % amygdalin	Alk	Alk	Alk	Alk
KNO_3, mineral salts medium without $CaCO_3$, +, (6)				
1 % sodium mucate	Alk	Alk	Alk	Alk
1 % sodium 1-mannonate	Alk	Alk	Alk	Alk
1 % sodium d-saccharate	Alk	Alk	Alk	Alk
KNO_3, mineral salts medium + $CaCO_3$ +, (6)				
1 % calcium 5 ketogluconate	N	N	0	0
1 % calcium d-gluconate	Alk	Alk	Alk	Alk
Nitrate glycerol agar (19)	0?	+?	0	0

Symbols: A, acid; Alk, alkaline; N, no change; s.z., serum zone; 0, no growth; +, growth

* Strains of rhizobia from *S. macrocarpa* and *S. longifolia*.

** Strains of rhizobia from *S. grandiflora*, *S. tomentosa*, *S. aculeata* and *S. sesban*.

related strains.

R. egumiosarum	*R. phaseoli*	*R. japonicum*	Cowpea rhizobia	*R. lupini*	*Agrobacterium*	
					radiobacter	*tumefaciens*
8	4	8	8	8	8	10
A s.z.	A s.z.	Alk no s.z.	Alk no s.z.	Alk no s.z.	browning	Alk, coagulation
—	—	—	—	—	++	0
—	—	+	+	+	—	+
0	0	0	0	0	+	+
0	0	0	0	0	+	+
0	0	0	0	0	++, P	+
0	0	0	0	0	++, P	+
0	0	0	0	0	++, P	+++
0	0	0	+	0	++, P	+
0	0	0	0	0	++, P	+
0	0	0	0	0	++, P	+
—	—	—	—	—	+++	+
Alk	Alk	Alk	Alk	Alk	A	A
Alk	Alk	Alk	Alk	Alk	A	A
Alk	Alk	Alk	Alk	Alk	A	A
Alk	Alk	Alk	Alk	Alk	N	N
Alk	Alk	Alk	Alk	Alk	A	A
Alk	Alk	Alk	Alk	Alk	A	A
0	0	0	0	0	Alk	Alk
Alk	Alk	Alk	Alk	Alk	A	A
0	0	0	+	0	++	++

, growth but negative action; P, pellicle.

TABLE 3.

Tolerance of crystal violet in peptone-sucrose agar by sesbania rhizobia and related organisms.
(*See Table 1 for strain numbers not listed)

Organisms Tested	Concentration of Crystal Violet					
	1 : 2,500	1 : 5,000	1 : 7,500	1 : 10,000	1 : 20,000	1 : 30,000
S. macrocarpa						
762, 763, 1050	—	—	—	+	++	++
764	—	+	++	++	++	++
765	—	—	—	—	+	++
773	—	—	+	++	++	++
774	—	—	—	—	—	+
775	—	—	—	++	++	++
S. longifolia						
776, 777, 1051, 1052	+	++	++	++	++	++
S-1, S-9, S-10	—	—	+	++	++	++
S-2, S-5, S-6	—	—	—	+	++	++
S-3	—	—	—	—	—	+
S-4	—	+	+	++	++	++
S-7, S-8	—	—	—	—	+	++
S. grandiflora						
780	—	+	++	++	++	++
781	—	—	—	—	—	+
*5 strains	—	—	—	+	++	++
S. aculeata						
789	—	—	—	+	++	++
791	—	—	+	++	++	++
S. sesban						
3 strains	—	—	—	+	++	++
S. tomentosa						
768, 771	—	—	—	—	—	+
769	—	—	—	+	++	++
772	—	—	+	++	++	++
786	—	—	—	—	+	++
R. meliloti						
101, 120	—	—	—	—	—	—
122	—	—	+	++	++	++
R. trifolii						
201, 203, 208, 214	—	—	—	+	+	+
204, 207, 209, 210	—	—	—	—	—	—
R. leguminosarum						
8 strains	—	—	—	+	+	+
R. phaseoli						
4 strains	—	—	—	+	+	+

TABLE 3 - continued.

Organisms Tested	Concentration of Crystal Violet					
	1 : 2,500	1 : 5,000	1 : 7,500	1 : 10,000	1 : 20,000	1: 30,000
R. japonicum						
8 strains	—	—	—	—	—	—
Cowpea rhizobia						
8 strains	—	—	—	—	—	—
R. lupini						
8 strains	—	—	—	—	—	—
A. radiobacter						
8 strains	++	++	++	++	++	++
A. tumefaciens						
10 strains	+	++	++	++	++	++

the various strains to concentrations of crystal violet added to the peptone-sucrose medium described by HOPKINS *et al.* (11). This medium was adjusted to pH 7.0. The observations as recorded after 2-week incubations are noted in Table 3.

With the exception of the strains from *S. longifolia*, marked differences in tolerance were obtained between all the rhizobia and those of *A. radiobacter*. On the medium of SMITH (18) containing 1 : 80,000 crystal violet, colonies of *A. radiobacter* were raised, smooth, glistening and with opaque centers and transparent edges, whereas those of rhizobia were small and deeply colored. Differentiation between the strains of *A. tumefaciens* and *A. radiobacter* on this medium was not feasible.

These results prompted an inquiry into the possibility that a relationship exists between resistance of rhizobia to crystal violet and gum formation. Accordingly, the method of HOPKINS, PETERSON and FRED (11) was used to determine the amounts of gum produced by 17 selected strains of the sesbania rhizobia. After the cultures had grown two weeks in peptone-sucrose medium, the extracellular gum was removed from the cells by a Sharples centrifuge at 42,000 r.p.m., the supernatants were concentrated in a vacuum oven at 60° C.; two volumes of acetone were added and the mixtures allowed to stand for 24 hours. The precipitated gum was then filtered by suction through a weighed Gooch asbestos filter according to the method of RAJAGOPALAN (14), dissolved in warm water, precipitated again and subsequently filtered. Final weights of the gum were obtained after drying the residue to a constant weight at 100° C. These results are shown in Table 4.

TABLE 4.

Gum production by sesbania rhizobia on peptone-sucrose medium.

Rhizobial Strain	Dry Wt. (mg) of Gum (per 100 ml)
S. macrocarpa	
763	67.3
765	38.7
773	31.3
774	36.2
1050	39.3
S. longifolia	
776	45.1
777	36.0
1051	62.9
1052	43.1
S. grandiflora	
780	52.8
781	46.7
782	43.3
785	61.6
S. aculeata	
791	73.8
S. sesban	
792	67.8

The relationship between tolerance of crystal violet and gum production is obtained by comparing the data contained in Tables 3 and 4. Insignificantly small amounts of gum were produced by the slow-growing strains of *R. japonicum*, *R. lupini* and the cowpea rhizobia; these data are omitted from Table 4 for the sake of brevity. In the light of this fact, it may be concluded that gum-forming rhizobia cultures are among the most resistant to crystal violet; yet, as shown in Tables 3 and 4, it does not obtain quantitatively that the heaviest gum-producing strains are necessarily the most resistant to crystal violet.

Summary.

1. All the sesbania strains employed in these studies were strikingly similar to other well-known rhizobia as shown by a wide variety of tests involving a diverse collection of mineral salts media containing a variety of carbon and nitrogen sources.

2. In comparative tests the 39 strains of sesbania rhizobia are divided into two well-defined groups. Those from *S. macrocarpa* and *S. longifolia*, designated as Group 1, were culturally similar to the fast-growing rhizobia from clover, peas and beans. These strains produced mucilaginous colonies on all agar media, did not grow in HOFER's alkaline media, veal infusion broth, CLARK and LUB's, KOSER's uric acid and ZOBELL's media and did not reduce nitrate. Others from *S. grandiflora*, *S. tomentosa*, *S. aculeata* and *S. sesban*, comprising Group 2, produced compact opaque colonies, grew in the above media and reduced nitrate slowly.

3. Question is raised that Congo red agar is a completely reliable medium for the separation of rhizobia from closely allied bacteria, as judged by dye absorption.

4. The strains of sesbania rhizobia showed considerable variation in their abilities to tolerate crystal violet in a peptone-sucrose medium. The majority of fast-growing strains of the designated rhizobial species did not grow in concentrations greater than 1 : 10,000 crystal violet; none of the slow-growing strains tolerated concentrations above 1 : 30,000. All the strains of species in the genus *Agrobacterium* resisted a 1 : 2,500 concentration.

5. Evidence is presented that a relationship exists between tolerance of rhizobial strains to crystal violet dye in media and their abilities to produce gum.

6. Various cultural tests are described which permit reliable differentiations between rhizobia and related bacteria, exemplified by diverse strains of *A. tumefaciens* and *A. radiobacter*.

Literature.

1. W. A. ALBRECHT and T. M. MCCALLA, J. Bact. **34**, 455, 1937. – 2. ETHEL K. ALLEN and O. N. ALLEN, Bact. Rev. **14**, 273, 1950. – 3. O. N. ALLEN, Experiments in Soil Bacteriology, Burgess Publishing Co., Minneapolis, Minn., U. S. A., 126 pp., 1949. – 4. C. S. BRYAN, Soil Sci. **45**, 185, 1938. – 5. M. DAMODARAN and M. SRINIVASAN, Proc. Indian Acad. Sci. **2B**, 337, 1935. – 6. C. E. GEORGI and JANE M. ETTINGER, J. Bact. **41**, 323, 1941. – 7. J. O. HARRIS, Unpublished Master of Science thesis, 86 pp., University of Hawaii, Honolulu, T. H., 1941. – 8. A. W. HOFER, J. Bact. **25**, 56, 1933. – 9. A. W. HOFER, J. Amer. Soc. Agron. **27**, 228, 1935. – 10. A. W. HOFER, J. Bact. **41**, 193, 1941. – 11. E. W. HOPKINS, W. H. PETERSON and E. B. FRED, J. Amer. Chem. Soc. **52**, 3659, 1930. – 12. S. A. KOSER, J. Infect. Dis. **23**, 377, 1918. – 13. T. MATSUOKA, Japanese patent 35952, March 11, 1920 (see Chem. Abs. **15**, No. 1406, 1921). – 14. T. RAJAGOPALAN,

Indian J. Agr. Sci. **8**, 331, 1938. – 15. M. S. Raju, Zentr. Bakt. II, **94**, 337, 1936. – 16. A. J. Riker, W. M. Banfield, W. H. Wright, G. W. Keitt and H. E. Sagen, J. Agr. Res. **41**, 507, 1930. – 17. A. J. Riker, Mary M. Lyneis and S. B. Loche, Phytopath. **31**, 964, 1941. – 18. N. R. Smith, J. Bact. **15**, 20, 1928. – 19. N. R. Smith and H. Humfeld, J. Agr. Res. **41**, 97, 1930. – 20. C. E. ZoBell and Catherine B. Feltham, J. Bact. **28**, 169, 1934.

(From the Department of Bacteriology, University of Wisconsin, Madison 6, Wisconsin).

NODULATION STUDIES WITH SPECIAL REFERENCE TO STRAINS ISOLATED FROM *SESBANIA* SPECIES ¹)

by

M. D. JOHNSON and **O. N. ALLEN** ²)

(Received July 31, 1951).

The ability of rhizobia to form nodules on the roots of certain leguminous plants is one, if not the most important, way of differentiating between rhizobia *per se.* Since the concept of early workers that all rhizobia comprised one species capable of nodulating all leguminous plants, about 22 cross-inoculation groups of plants have been established (3), yet only 6 *Rhizobium* species are recognized (5, 9). Among the plants in each of these groups the strains of rhizobia are more or less specific in their infective abilities. Within recent years certain data (18, 19) have cast doubt upon the validity of cross-inoculation groupings.

The present study reports the results of plant inoculation tests conducted over a three-year period in which 39 strains of rhizobia from 6 species in the genus *Sesbania* and 52 strains from plants contained in 7 of the commonly recognized cross-inoculation groups were used as inocula. A better understanding of the infective abilities of sesbania rhizobia is warranted in view of the importance of *Sesbania* species in agriculture and because of certain divergencies of opinion as to their plant host range.

METHODS.

The following species of the genus *Sesbania* were used as host plants:

(a) *S. macrocarpa* Muhl. and Rafin. — an annual species which is

---

¹) Published with the approval of the director, Wisconsin Agricultural Experiment Station.

²) Research assistant and Professor of Bacteriology, respectively.

native to and widely distributed in many temperate regions including southern United States and northern Mexico. On the alluvial soils of the southwestern United States this plant is commonly known as Colorado River hemp. Seeds of this species were obtained from the Stanley Seed Company, Brawley, California.

(b) *S. sonorae* Rydb. — a species similar to the above, but because of its shorter stem and small black seeds it is considered by certain plant taxonomists as a form of *S. macrocarpa*. Bona fide seed material of this species was obtained from Javier A. Gomez, Magdalena, Sonora, Mexico.

(c) *S. exaltata* (Rafin.) Cory — a plant bearing comparatively large, light brown seeds and commonly known as Golden Sesbania. This seed material was identified in 1950 by experts in the United States Department of Agriculture for the Advance Seed Company, Phoenix, Arizona.

(d) *S. grandiflora* Poir. — a large shrub or tree widely distributed in many tropical and subtropical areas.

(e) *S. tomentosa* Hook. and Arn. — an erect, flowering shrub indigenous to the Hawaiian Islands.

The seed lots of the last-named species were obtained through the courtesy of William Crosby, Wailupe, Oahu, Territory of Hawaii.

In addition, the following well-known leguminous plants were included: alfalfa (*Medicago sativa* L.), red clover (*Trifolium pratense* L.), Alaska field peas (*Pisum sativum* L.), vetch (*Vicia sativa* L.), soybean (*Glycine max* (L.) Merr.), yellow lupine (*Lupinus luteus* L.) and cowpea (*Vigna sinensis* Endl.).

All plant inoculation tests were conducted in cotton-stoppered glass jars and also in open earthenware jars. Sterile white quartz sand served as the growth substrate. When the larger plants could not be contained *in toto* in the glass jars because of their growth habits, the surface-sterilized seeds were placed on the surface of the moistened sterile quartz sand which filled the jars within several inches of their tops. At the proper times sterile cotton was placed around the stems of the plants to form a firm plug. This measure was taken so as to prevent possible root contamination. Otherwise the plants were grown completely within the jars which were similarly plugged with sterile cotton over which loose fitting paper cups were inverted. The plants in the open jars were also grown in sterile white quartz sand over which was added a one-inch layer of sterile pebble gravel. All plants were watered at weekly intervals with sterile Crone's modified nitrogen-free nutrient solution (1) and when necessary with sterile distilled water.

Prior to planting, the seeds were surface-treated with a 1 : 1,000 solution of mercuric chloride for 10—30 minutes, depending on their size and contour, and dried immediately thereafter in sterile petri dishes. The efficiency of the surface-sterilization treatment was tested by germinating all, or an appreciable aliquot, of the seeds on the surface of sterile yeast water mannitol agar, as described by ASH and ALLEN (4). When contamination occurred on the agar surface or was evidenced on the germinating seeds, the particular lot of seed was discarded and an additional batch of seeds was again treated and tested as described above. This procedure was repeated until no contamination occurred. After sterilization the seedlings were planted under aseptic conditions in their respective containers.

The inocula comprised 2—4 ml of a 4-day-old broth culture of the respective rhizobia for each jar which contained 10—15 seedlings. Duplicate or more jars of each plant species were seeded with each strain. Twenty per cent of the total number of each of the closed and open jars were seeded but not inoculated. These served as controls. The results reported here were obtained from replicated experiments. Other details pertinent to the installation of these experiments are well described elsewhere (1).

The times of harvest varied with the species owing to the different growth habits of the many plants tested. The durations of growth ranged between 44 days (*Phaseolus vulgaris*) and 83 days (*S. grandiflora*). The tests were conducted during the early fall and spring months when growing conditions in the greenhouse were optimum.

Contamination was rare, yet it is fitting to note that when any evidence of contamination was obtained, the entire set was discarded. The jars were randomized on the greenhouse benches for the purpose of equalizing environmental conditions. At the times of harvest the plants from duplicate jars of the same series were combined, dried at 37° C. for 5—7 days, then at 100° C. for 6 hours and the dry weights recorded. The dried plants were pulverized in a Wiley mill and adequate portions were used for the determination of total nitrogen by the Kjeldahl method followed by the distillation process, as outlined by ALLEN (1). A minor variation in this technique comprised the use of Hengar granules (selenium-coated granules) instead of copper selenite.

TA

Cross-inoculatio

(*Host plants

Inocula					Tes
	S. macro-carpa	*S. grandi-flora*	*S. tomen-tosa*	*S. exaltata*	*S. sonorae*
**S. macrocarpa*					
762	+, E	+, I	+, E	+, E	+, E
763	+, I	+, I	+, E	+, E	+, E
764	+, I	+, I	+, E	+, I	+, I
765	+, I	+, I	+, I	+, I	+, I
773	+, E	+, I	+, E	+, E	+, E
774	+, E	+, I	+, E	+, E	+, E
775	+, I	+, I	+, I	+, I	+, I
1050	+, E	+, I	+, I	+, E	+, E
**S. grandiflora*					
7 strains	+, E	+, E	+, E	—, E	+, E
**S. tomentosa*					
5 strains	+, I	+, I	+, E	+, E	+, E
**S. longifolia*					
776	+, E	—, I	+, E	+, E	—, E
777	+, E	—, I	+, E	+, E	+, E
1051	+, I	—, I	+, I	+, I	+, I
11 strains	+, E	+, E	—, E	+, E	+, E
**S. aculeata*					
2 strains	+, E	+, E	+, E	+, E	+, E
**S. sesban*					
3 strains	+, E	+, E	+, I	+, E	+, E
R. lupini					
8 strains	—	—	—	—	—
R. japonicum					
8 strains	—	—	—	—	—
R. trifolii					
8 strains	—	—	—	—	—
R. meliloti					
8 strains	—	—	—	—	—
R. phaseoli					
4 strains	—	—	—	—	—
Cowpea rhizobia					
601	—	—	—	—	—
602	—	—	—	—	—
6 strains	—	—	—	—	—
R. leguminosarum					
8 strains	—	—	—	—	—

Symbols: +, nodules present; —, nodules absent; E, effectiveness; I, ineffectiveness.

1.
ficities.
isolates)

ts							
L. iteus	*Glycine max*	*T. pratense*	*M. sativa*	*P. vulgaris*	*Vigna sinensis*	*Pisum sativum*	*Vicia sativa*
—	—	—	—	+, I	+, I	—	—
—	—	—	—	+, I	+, I	—	—
—	—	—	—	+, I	+, I	—	—
—	—	—	—	+, I	+, I	—	—
—	—	—	—	+, I	+, I	—	—
—	—	—	—	+, I	+, I	—	—
—	—	—	—	+, I	+, I	—	—
—	—	—	—	+, I	+, I	—	—
—	—	—	—	+, I	+, I	—	—
—	—	—	—	+, I	+, I	—	—
—	—	—	—	+, I	+, I	—	—
—	—	—	—	+, I	+, I	—	—
—	—	—	—	+, I	+, I	—	—
—	—	—	—	+, I	+, I	—	—
—	—	—	—	+, I	+, I	—	—
—	—	—	—	+, I	+, I	—	—
+, E	—	—	—	—	—	—	—
—	+, E	—	—	—	—	—	—
—	—	+, E	—	—	—	—	—
—	—	—	+, E	—	—	—	—
—	—	—	—	+, E	—	—	—
—	+, I	—	—	—	+, E	—	—
—	+, I	—	—	—	+, E	—	—
—	—	—	—	—	+, E	—	—
—	—	—	—	—	—	+, E	+, E

RESULTS.

The results of the plant inoculation tests are contained in Table 1. The data obtained with the sesbania rhizobia are recorded in detail inasmuch as the plant responses were somewhat diversified. In the other plant series where the reactions were identical, the results have been summarized relative to the total number of strains used. Itemization of the dry weights and nitrogen analyses are omitted for the sake of brevity. The reader is referred to an earlier paper (11) for strain identifications.

Ineffectiveness, "I," is defined here as the fixation of little or no nitrogen without any otherwise adverse effect on the host plant. The nodules formed were small, white and profusely scattered throughout the root systems; the plants were spindly, greatly retarded in growth and showed definite signs of nitrogen deficiency. None of the strains was parasitic because the plants nodulated by them did not show less vigor, growth and nitrogen content than those of the noninoculated controls. Effectiveness, "E," was considered synonymous with the fixation of considerable nitrogen as evidenced by a luxuriant dark green foliage. While, admittedly, certain gradations in effectiveness were apparent among the desirable strains, no attempt is made here to subdivide this category. The effective response was always accompanied by the presence of large pink or reddish nodules located primarily on the upper root systems.

Results obtained with the plants grown in the closed and open earthenware jars were identical relative to nodule occurrence. As was to be expected, however, the foliage was more luxuriant, nodulation was more abundant and the growth of the plants was nearer normal in the open jar series. In no instance were the plants nodulated in one series and not nodulated in the jars of the parallel series.

The results obtained in these many plant inoculation tests are emphatic. Considerable variation was noted in the infective and effective ranges of the sesbania rhizobia on the various *Sesbania* species. Sixteen of these 39 strains failed to benefit the growth of *S. grandiflora*; only 4 strains failed to enhance the growth of *S. exaltata* and *S. sonorae*. Notably, all of the strains isolated from *S. grandiflora* and *S. aculeata* benefited the growth of the 5 *Sesbania* species. Of equal interest is the fact that none of the strains from

S. tomentosa was effective on *S. macrocarpa* and *S. grandiflora*, yet these same strains were highly effective on the other three species employed.

Cross-infection of the strains on the other test plants was not common. Despite the formation of nodules and ineffective responses of the 39 sesbania rhizobia on bean and cowpea plants, none of the 8 strains of rhizobia from each of these latter plants nodulated the *Sesbania* species. Only 2 of the 8 cowpea rhizobia produced nodules on soybeans. In all other instances the rhizobial strains were specific for the plants contained in their respective cross-inoculation groups.

In additional tests conducted in the same manner as heretofore described, all the sesbania strains failed to nodulate *Cyamopsis tetragonoloba* (L.) Taub., *Erythrina indica* Lam., *Crotalaria anagyroides* H. B. K., *Indigofera suffruticosa* Mill., *Canavalia campylocarpa* Piper and *Phaseolus lathyroides* Linn. Rhizobia from these species were not available for use as inocula in reciprocal tests. Irregular nodulation of these last-named species by diverse cowpea rhizobia has been reported earlier by ALLEN and ALLEN (2).

DISCUSSION.

Available data obtained by other investigators indicate certain degrees of promiscuity in the infective abilities of rhizobia from *Sesbania* species. Tests employing 5 strains isolated from dhaincha (*S. aculeata* Poir.) were described in 1936 by RAJU (13). These strains produced slight nodulation of cowpea (*V. sinensis*), gram bean (*Dolichos biflorus* L.) and *Phaseolus trilobus* Ait. but failed to nodulate alfalfa, red clover, peas, soybeans, the common bean, Wood's clover (*Dalea alopecuroides* Millsp.), math bean (*Phaseolus acontifolius* Jacq.) and black locust (*Robinia pseudoacacia* L.). In reciprocal tests the rhizobia from these latter plants did not nodulate dhaincha. On this premise RAJU proposed that *Sesbania* species and their microsymbionts should constitute a new bacteria: plant group. In 1938 BRISCOE and ANDREWS (6) tentatively defined the "Sesban cross-inoculation group" based on the finding that bean and cowpea rhizobia effectively nodulated *S. aculeata*, although in reciprocal tests sesbania strains formed ineffective nodules on bean but no nodules on cowpeas. This terminology was soon refuted on the basis of priority by RAJU (14), who emphasized the name "dhaincha bacterial-plant group"; four species were included: *S. speciosa*, *S.*

exaltata, *S. grandiflora* and *S. aculeata*. Although the work of Harris (10) was in general agreement with these findings, he opined that the cross-inoculation status of this genus should be provisional only and subject to further investigations. Appreciation of these results was somewhat complicated by the work of Wilson (18), who reported that rhizobia from *S. macrocarpa* nodulated 85 of 160 plant species tested by him. At a later date (19) he proposed the abandonment of all cross-inoculation groupings.

An analysis of the experimental data reported here poses several matters for consideration especially in the light of those of the aforementioned investigators. While, admittedly, these results (Table 1) are in general agreement with those of Raju (13), Briscoe and Andrews (6) and Harris (10) and bear little, if any, resemblance to those of Wilson (18), the establishment of another cross-inoculation group at the present time hardly seems warranted. A valid reason for this opinion stems from the fact that reciprocal cross-infections of *Sesbania*, bean and cowpea plants by the respective rhizobia were not obtained. Nonetheless, that the sesbania rhizobia bear a close relationship in infective powers to cowpea and bean rhizobia appears well defined. Observations showing cultural and physiological patterns of sesbania rhizobia are contained in an earlier paper (11).

In essence, our original concept of cross-inoculation grouping has been somewhat vitiated in recent years by reports showing lack of mutual interchangeability by rhizobia upon plants in the same group (2, 7, 8), nodulation of plants in different groups by the same rhizobia (15, 18) and other irregularities (17, 20). Underlying many of these contributions is evidence that pollination (16) and genetic (12) patterns of the plant play dominant roles in the nodulation process. Accordingly, the plant can be viewed no longer merely as a reflector of the stimuli brought about by a specific rhizobial strain. It remains, therefore, for investigators to confirm or deny host specificity as a primary taxonomic character before confusion is compounded by the formulation of additional cross-inoculation groups which increase, rather than simplify, certain existent problems.

From another point of view, it may well be borne in mind that the *Leguminosae* comprise the second largest family of the seed plants, yet only about 10 per cent of the conservative estimate of 10,000 species have been mentioned in nodule bacteria studies. Our understanding of these bacteria today is based on comparative studies of relatively few strains obtained from species contained in only about a dozen genera (3). It follows that the cataloguing of all strains of rhizobia within a few specific limitations hardly promises to be a simple matter. From an evolutionary point of view it is logical to assume that certain rhizobia have reached an adaptive peak in their infective abilities and are therefore restricted to closely related plant species. This is evidenced somewhat by certain stabilities of well-known strains in their host range, their capacity to aid their host plants and, in a related way, by constancy in their physiological characteristics. Were it possible to know the detailed histories of these strains, one might find that they had become specialized as a result of prolonged adaptation to certain hosts or because of other extenuating circumstances. At the other extreme, it is equally probable that many strains of rhizobia have apparently not reached a peak of stability but exist more or less as innumerable, intergrading forms whose infective abilities for leguminous plants are more versatile. The sesbania rhizobia and others are seemingly examples of this type.

Needless to say, all attempts to identify and separate the rhizobia *per se* by conventional laboratory tests have yielded few promising leads. At the present time the taxonomic status of a strain is best defined in terms of the host plant from which the parent nodule was obtained and relative to the plant species upon which it forms nodules. The limitations surrounding these criteria are obvious, especially since the key to the master reaction explaining infection of leguminous plants by rhizobia has not yet been found. As our techniques and methods of analyzing this symbiotic association are refined, so will our knowledge enlarge in scope and our understanding of the interrelationship become more valid. Until such time when a more complete understanding prevails, conservatism in the artificial separation of these bacteria is warranted.

Summary.

Nodulation tests are described in which 39 strains of rhizobia isolated from 6 *Sesbania* spp., 52 strains of 6 well-recognized rhizobial species and cowpea bacteria were employed. The results are briefed as follows:

1. The strains of sesbania rhizobia showed marked degrees of host specificity on the *Sesbania* spp. tested.

2. All the sesbania rhizobia formed nodules and brought about ineffective responses in plant growth on bean and cowpea plants. None of the bean and cowpea strains produced nodules on the *Sesbania* spp.

3. The diverse strains of *R. meliloti*, *R. trifolii*, *R. phaseoli*, *R. leguminosarum*, *R. lupini* and *R. japonicum* produced nodules only on the plants contained in their respective bacteria: plant groups. Of the 8 strains of cowpea rhizobia employed, only 2 strains formed nodules on soybean plants.

4. No evidence is contained in this report that the present system of cross-inoculation grouping should be abandoned.

5. The plausibility of variations in infective power of the rhizobia is discussed.

Literature.

1. O. N. ALLEN, Experiments in Soil Bacteriology, Burgess Publishing Co., Minneapolis, Minn., U. S. A., 126 pp., 1949. – 2. O. N. ALLEN and ETHEL K. ALLEN, Soil Sci. **47**, 63, 1939. – 3. O. N. ALLEN and ETHEL K. ALLEN, Soil. Sci. Soc. Amer., Proc., **12**, 203, 1947. – 4. C. G. ASH and O. N. ALLEN, Soil Sci. Soc. Amer., Proc., **13**, 279, 1948. – 5. R. S. BREED *et al.*, Bergey's Manual of Determinative Bacteriology, 6th ed., 1529 pp., Williams and Wilkins Publishing Co., Baltimore, Md., U. S. A., 1948. – 6. C. F. BRISCOE and W. B. ANDREWS, J. Amer. Soc. Agron. **30**, 135, 1938. – 7. W. R. CARROLL, Soil Sci. **37**, 117, 1934. – 8. MARIE E. CONKLIN, Soil Sci. **41**, 167, 1936. – 9. E. B. FRED, I. L. BALDWIN and ELIZABETH McCOY, Root Nodule Bacteria and Leguminous Plants, Univ. Wis. Studies Sci. **5**, 343 pp., 1932. – 10. J. O. HARRIS, Unpublished Master of Science thesis, 86 pp., University of Hawaii, Honolulu, T. H., 1941. – 11. M. D. JOHNSON and O. N. ALLEN, Antonie van Leeuwenhoek **18**, 1, 1952. – 12. P. S. NUTMAN, Heredity **3**, 263, 1949. – 13. M. S. RAJU, Zentr. Bakt. II, **94**, 249, 1936. – 14. M. S. RAJU, Science **88**, 300, 1938. – 15. J. J. REID, Unpublished Ph.D. thesis, 103 pp., University of Wisconsin, Madison, Wis., 1936. – 16. J. K. WILSON, J. Amer. Soc. Agron. **31**, 159, 1939. – 17. J. K. WILSON, J. Amer. Soc. Agron. **31**, 934, 1939. – 18. J. K. WILSON, Cornell Univ. Agr. Exp. Sta., Mem. **221**, 44 pp., 1939. – 19. J. K. WILSON, Soil Sci. **58**, 61, 1944. – 20. J. K. WILSON, Cornell Univ. Agr. Exp. Sta., Mem. **272**, 21 pp., 1946.

(Institut de Bactériologie de l'Université de Liège).

STREPTOMYCINE ET LYSOZYME

par

MAURICE WELSCH

(Reçu le 19 Octobre 1951).

L'agglutination des bactéries par la streptomycine, d'abord signalée par LINZ (9), a été étudiée, indépendamment, par GROS, MACHEBOEUF, RYBAK et LACAILLE (7). Ces derniers auteurs ont constaté que les antigènes O complets, ou, plus simplement, leur polyoside constitutif, inhibe l'agglutination streptomycinique. Nous avons rapporté, d'autre part, que diverses bactéries, sensibles à l'action lytique du lysozyme, ne sont pas, ou peu, agglutinées par la streptomycine (18). Cette inagglutinabilité nous parait attribuable à la présence, chez ces bactéries, du mucopolysaccharide (5, 8, 11, 12, 14) qui constitue le substrat spécifique du lysozyme.

Au cours de ces recherches, nous avons observé que la streptomycine exerce, à son tour, une certaine action inhibitrice sur la clarification des suspensions microbiennes par le lysozyme.

A. MATÉRIEL.

1. Souches. Nous avons utilisé quatre souches microbiennes distinctes: *Micrococcus lysodeïkticus*, *Sarcina lutea*, *Bacillus megatherium* 899 (lysogène) et *B. megatherium* 36 (mutilat sensible au bactériophage).

En outre, par les méthodes que nous avons utilisées et décrites à propos de *Staphylococcus aureus* (17), nous avons isolé, de chacune des souches précitées, un variant ,,totalement résistant'' à la streptomycine (''first-step mutant''), c'est à dire capable de croitre en présence d' antibiotique à la concentration de 200 γ/ml, ou davantage.

2. Streptomycine. Nous avons utilisé, indifféremment et avec les mêmes résultats, soit le complexe streptomycine-$CaCl_2$ (Merck), soit le sulfate de streptomycine (Pfizer).

3. Lysozyme. Nos essais préliminaires ont été effectués avec une simple solution au 1/5 de blanc d'oeuf. Nos résultats ont été étendus ensuite au moyen de lysozyme pur, cristallisé [1]).

B. Observations expérimentales.

1. Action du lysozyme sur les suspensions microbiennes. Une clarification pratiquement complète des suspensions microbiennes, en eau distillée, à 37°C., a été obtenue, en 30 minutes ou moins, avec une concentration de lysozyme de 100 γ/ml environ. Dans les mêmes conditions, mais en solution NaCl 1 %, la clarification est obtenue avec une concentration de 5 γ/ml, ou même moins.

On sait que la présence de sels inhibe la formation de complexes entre la streptomycine et diverses substances, telles que les acides nucléiques, par exemple; qu'elle dissocie ces complexes lorsqu'ils se sont formés en absence de sels; qu'elle empêche l'agglutination streptomycinique des bactéries (3, 15, 16). C'est pourquoi, nous avons cherché quelle concentration minimale de NaCl permettait encore une bonne activité du lysozyme. Nous avons ainsi constaté que les suspensions microbiennes, en solution NaCl 0.1 %, étaient clarifiées, dans les conditions ci-dessus précisées, avec des concentrations en lysozyme de l'ordre de 10 γ/ml. Sauf indication contraire, nous avons fait tous nos essais dans ces conditions.

L'activité du lysozyme se manifeste, non seulement par la clarification de la suspension bactérienne, mais encore par une considérable augmentation de la viscosité du lysat (6).

Du point de vue microscopique, les cellules qui ont subi l'action du lysozyme perdent leur affinité pour les colorants usuels et sont difficiles à déceler par les méthodes habituelles. Par contre, les colorations par contraste (encre de Chine, etc.) permettent de mettre en évidence des "ghost-cells" conservant la forme générale des éléments intacts (1).

Dans le cas de *M. lysodeïkticus* et de *S. lutea*, nous avons mis en évidence, avant que la désintégration cellulaire ne soit trop avancée, de petites granulations basophiles, qui donnent une réaction de Feulgen positive et peuvent, par conséquent, être considérées comme des noyaux. Ces observations confirment celles de Caselli

---

[1]) Cette préparation, provenant du laboratoire de Mr Cl. Fromageot, nous a été fournie par Mr E. Nihoul que nous remercions vivement.

(2), de PETRONELLI (13), de CUTINELLI et LA MANNA (4). Nous n'avons pu mettre ces éléments en évidence dans les lysats des souches de *B. megatherium* utilisées.

Dans le cas particulier de *M. lysodeïkticus*, nous avons noté un stade particulier, au cours duquel ces noyaux, généralement groupés par paires, présentent un état de gonflement extrême, en même temps que leur basophilie diminue fortement.

Les variants résistants à la streptomycine se sont comportés vis à vis du lysozyme exactement comme les souches-mères.

2. Action de la streptomycine sur les suspensions microbiennes. L'addition de streptomycine, à raison de 1000 γ/ml, aux suspensions microbiennes étudiées que l'on maintient ensuite à 37°C., n'a provoqué une légère agglutination que dans deux cas; à savoir: avec les deux souches de *B. megatherium* streptomycino-resistantes. Il convient de dire, cependant, que l'addition de l'antibiotique provoque une très légère augmentation de la valeur du trouble des suspensions (mesurée au néphélomètre de Pulfrich). Cette augmentation ne dépasse toutefois pas 10% de la valeur du trouble initial.

La morphologie microscopique des bactéries soumises à l'action de la streptomycine n'est guère modifiée. Notons, pourtant, que les paires nucléaires, que l'on distingue fréquemment dans les cellules de *M. lysodeïkticus* (sensible ou résistant à la streptomycine), en suspension aqueuse ou saline diluée, apparaissent plus nettement en milieu streptomyciné. Cette netteté plus grande des noyaux n'a pas été observée avec les autres souches étudiées.

3. Action de la streptomycine sur les suspensions microbiennes lysozymées. Ainsi que l'ont déjà signalé LINZ et LECOCQ d'abord (10), nous-même ensuite (18), l'addition de streptomycine aux suspensions fraichement lysées y provoque une précipitation plus ou moins abondante. On peut séparer le lysat en un culot de débris microbiens et un liquide surnageant clair, visqueux. La streptomycine (1000 à 5000 γ/ml) provoque un précipité dans ce liquide surnageant et une agglutination des débris sédimentés repris en eau distillée.

L'examen microscopique du précipité, obtenu avec les liquides surnageants de *M. lysodeïkticus* et *S. lutea*, montre un matériel amorphe, peu basophile, colorable en rose pâle et de façon diffuse par la réaction de Feulgen. L'agglutinat, obtenu avec les culots de centrifugation, est constitué de grains sphériques basophiles, tous identiques, Feulgen-positifs et de matériel amorphe, également colorable, mais en plus pâle, par la réaction de Feulgen.

Si l'addition de streptomycine est différée de plusieurs heures, on trouve de moins en moins de noyaux reconnaissables.

Le précipité, obtenu avec les liquides surnageants des souches de *B. megatherium*, est abondant et s'agglomère en filaments épais et visqueux. A l'examen microscopique, ces trainées filamenteuses de matériel amorphe sont basophiles et fortement Feulgen-positives. Les agglutinats, obtenus avec les culots de centrifugation des lysats de ces mêmes organismes, fournissent des images microscopiques analogues.

Les variants streptomycino-resistants se comportent comme les souches-mères correspondantes.

4. Action simultanée de la streptomycine et du lysozyme sur les suspensions microbiennes. Les suspensions bactériennes, additionnées de streptomycine, sont portées à 37°C., pendant 30 minutes. On y ajoute ensuite le lysozyme et on prolonge l'incubation à 37°C. pendant 30 nouvelles minutes. Dans quelques expériences, le lysozyme a été introduit dans les suspensions 24 heures après la streptomycine: les résultats obtenus sont restés identiques.

La clarification des suspensions en eau distillée est totalement

TABLE 1.

Inhibition par la streptomycine de l'action clarifiante du lysozyme sur des suspensions aqueuses de *M. lysodeïkticus* et *S. lutea.*

Concentration de la streptomycine γ/ml	*M. lysodeïkticus*	*S. lutea*
	Trouble en % du trouble initial, après 30 minutes à 37° C. en présence de blanc d'oeuf à 1/500	
5000	103	105
1000	82	97
500	82	94,5
250	71	85
125	61,5	65,5
62	53	34
31	39	9,7
16	21,8	8,1
8	6,6	8,3
0	5,7	6,2

inhibée par la présence de 5000 γ/ml streptomycine (table 1). Elle n'est que partiellement entravée par cette concentration d'antibiotique en solution NaCl 1 %; dans ces conditions, en effet, la valeur du trouble initial, après action du lysozyme, est diminuée de moitié, alors que la suspension témoin, sans streptomycine, est totalement clarifiée. En solution NaCl 0.1 %, une inhibition appréciable de la clarification est encore observée avec 500 à 1000 γ/ml d'antibiotique (table 2).

TABLE 2.

Inhibition par la streptomycine de l'action clarifiante du lysozyme cristallisé (10 γ/ml) sur une suspension en NaCl 0,1 % de *M. lysodeïkticus.*

Concentration de la streptomycine γ/ml	Trouble après 30 minutes à 37° C. en % du trouble initial
10000	73
5000	76
2500	70,5
1250	56
625	42
312	25
156	23
78	18
0	11

L'effet inhibiteur de la streptomycine ne peut être attribué à une modification du pH, qui, dans tous les cas est resté compris entre 5,8 et 7,2.

Les suspensions, traitées simultanément par le lysozyme et la streptomycine, dont la clarification est totalement ou même partiellement inhibée, n'acquièrent pas la haute viscosité, si manifeste, des lysats usuels. Pourtant, l'examen microscopique montre des altérations cellulaires considérables, non seulement dans les suspensions ayant subi une clarification partielle, mais aussi dans celles dont le trouble n'a pas varié.

Dans le cas de *M. lysodeïkticus* et de *S. lutea*, ces suspensions sont uniquement constituées de granules arrondis, fortement basophiles et Feulgen-positifs, beaucoup plus nets et plus abondants que ceux que l'on retrouve parfois dans les suspensions lysozymées en l'absence de streptomycine.

Dans le cas des *B. megatherium*, les colorations usuelles montrent des bâtonnets très amincis et des formes anormales gonflées, plus ou moins sphériques. A côté de ces bactéries déformées, on voit des éléments plus petits: granulations arrondies ou cylindriques, parfois en forme d'haltère, souvent disposées à la suite les unes des autres en filaments spiralés. La réaction de Feulgen positive montre que ces divers petits éléments peuvent être considérés comme des formations nucléaires.

Il semble donc que la streptomycine, en concentration suffisante, et d'autant mieux que le milieu est moins riche en sels, protège les noyaux de la désintégration générale qui suit, plus ou moins rapidement, l'action initiale du lysozyme. Les éléments de ces suspensions conservent, en effet, leur morphologie inaltérée pendant plusieurs heures, ou même jours.

Les suspensions microbiennes, traitées par le lysozyme en présence de streptomycine, peuvent être centrifugées. Elles fournissent un liquide clair surnageant, qui ne précipite pas, ou à peine, par addition de streptomycine. Elles donnent, d'autre part, un culot de centrifugation qui peut être repris dans l'eau. Nous nous sommes demandé si l'addition de sel à cette suspension d'éléments microbiens protégés par la streptomycine ne libérerait pas l'antibiotique et ne déclencherait pas ainsi une lyse instantanée ou, en tout cas, rapide. Contrairement à notre attente, l'addition de NaCl, de façon à obtenir une concentration finale de 5 %, n'a été suivie ni de clarification, ni de modification de l'aspect microscopique. Par contre, après cette addition de sel, la suspension, restée trouble, a instantanément acquis une viscosité élevée, comparable à celle des suspensions clarifiées par le lysozyme.

Les éléments dont la destruction est empêchée par la streptomycine se dissolvent rapidement après addition de NaOH jusqu'à pH 10 environ.

C. CONCLUSIONS.

1. Les espèces microbiennes sensibles au lysozyme sont inagglutinables, ou mal agglutinables, par la streptomycine.

2. Cette inagglutinabilité parait attribuable à la présence du mucopolysaccharide particulier à ces espèces.

3. Les variants streptomycino-résistants conservent leur sensibilité au lysozyme. Ils sont parfois plus agglutinables par la streptomycine que les souches-mères correspondantes.
4. La streptomycine exerce une action inhibitrice sur la clarification par le lysozyme des espèces microbiennes sensibles à cette substance.
5. L'efficacité de la streptomycine, à ce point de vue, est plus grande en eau distillée qu'en solution saline.
6. Cette action de la streptomycine est essentiellement attribuable à une prévention de la dissolution des noyaux bactériens.
7. Les suspensions soumises à l'action du lysozyme en présence de streptomycine ne montrent pas la haute viscosité des lysats usuels.
8. La streptomycine provoque la formation d'un précipité dans les suspension lysozymées. Ce précipité contient parfois des noyaux bactériens non altérés et toujours du matériel amorphe donnant une réaction de Feulgen positive.
9. Le matériel nucléaire est vraisemblablement pour une part importante dans la viscosité élevée des lysats.

Résumé.

Les microorganismes sensibles au lysozyme ne subissent pas l'agglutination par la streptomycine. Inversement, la streptomycine empêche la clarification des suspensions microbiennes par le lysozyme. Cet effet est attribuable à la prévention de la dissolution des noyaux.

Les mutants résistants à la streptomycine restent sensibles au lysozyme et se comportent, en présence de streptomycine plus lysozyme, comme les souches-mères.

Abstract.

Lysozyme-sensitive microorganisms are not subject to streptomycin-agglutination. Conversely, streptomycin inhibits the clarification of bacterial suspensions by lysozyme. This effect is mainly due to a prevention of nuclear dissolution.

Streptomycin-resistant mutants remain lysozyme-sensitive and behave towards streptomycin plus lysozyme as do parent-cells.

Références.

1. E. H. Boasson, J. Immunol. **34,** 281, 1938. – 2. P. Caselli, Boll. Soc. ital. Biol. sperim. **23,** 1176, 1947. – 3. S. S. Cohen, J. Biol. Chem. **168,** 511, 1947. – 4. C. Cutinelli et N. La Manna, Riv. Ist. sierot. ital. **25,** 1, 1950. – 5. L. A. Epstein et E. Chain, Brit. J. exp. Path. **21,** 339, 1940. – 6. A. Fleming, Proc. Roy. Soc. B. **93,** 306, 1922. – 7. F. Gros, M. Macheboeuf, P. Lacaille et B. Rybak, C. R. Acad. Sci. **228,** 720, 1949; Ann. Inst. Pasteur **77,** 246, 1949. – 8. C. Hallauer, Zentralbl. f. Bakt. **114,** 519, 1929. – 9. R. Linz, Ann. Inst. Pasteur **76,** 250, 1949. – 10. R. Linz et E. Lecocq, C. R. Soc. Biol. **144,** 305, 1950. – 11. K. Meyer et E. Hahnel, J. Biol. Chem. **163,** 723, 1946. – 12. K. Meyer, J. W. Palmer, R. Thompson et D. Khorazo, J. Biol. Chem. **113,** 479, 1936. – 13. A. Petronelli, Boll. Ist. sierot. Milan. **29,** 252, 1950.– 14. A. Pirie, Brit. J. exp. Path. **21,** 125, 1940. – 15. B. Rybak et F. Gros, Experientia **4,** 396, 1948. – 16. B. Rybak, F. Grumbach et F. Gros, Ann. Inst. Pasteur **77,** 237, 1949. – 17. M. Welsch, C. R. Soc. Biol. **143,** 1141, 1282, 1401, 1603, 1949; **144,** 432, 732, 981, 1142, 1950; Bull. Acad. Roy. Méd. Belg. **15,** 454, 1950. – 18. M. Welsch, C. R. Soc. Biol. **145**, 778, 1951.

(Aus dem Institut für landw. Bakteriologie und Gärungsbiologie, Eidg. Techn. Hochschule, Zürich).

UNTERSUCHUNGEN ÜBER DIE PHYSIOLOGIE DER WEINHEFEN. II. MITTEILUNG.

WACHSTUMSBEDINGUNGEN WEITERER AUXO-AUTOTROPHER, SCHWEIZERISCHER KULTURWEINHEFEN [1])

von

T. WIKÉN und **O. RICHARD**

(Eingegangen am 12. September 1951).

Seitdem Copping (3) entdeckt hatte, dass gewisse Hefe-Arten einen Zusatz kleiner Mengen ,,Bios"-Substanzen brauchen, um sich in einer synthetischen Nährlösung vermehren zu können, ist der Bedarf der Hefen an Wuchsstoffen Gegenstand zahlreicher Untersuchungen gewesen. Hinsichtlich der umfangreichen Literatur, welche auf diesem Gebiete vorliegt, sei auf die Arbeiten von Williams und Rohrman (14), Williams, Eakin und Snell (13), Leonian und Lilly (6), Burkholder (2), Emery, McLeod und Robinson (4), Atkin, Gray, Moses und Feinstein (1), Schopfer (10) sowie Menzinsky (8) verwiesen. Auf Grund dieser Untersuchungen ist die Hypothese aufgestellt worden, dass die wilden Hefen imstande sind, in einer synthetischen Nährlösung zu wachsen, welche nur Zucker und anorganische Salze enthält, während die Kulturhefen ein Substrat der erwähnten Zusammensetzung erst nach Zusatz eines oder einiger Wuchsstoffe assimilieren können. Man nimmt an, dass die in den Gärungsindustrien verwendeten *Saccharomyces*-Stämme, welche seit Jahren auf den im Laboratorium bereiteten Nährböden weitergezüchtet wurden, ursprünglich zur Assimilation einer nur Zucker und anorganische Salze enthaltenden Nährlösung fähig waren, dass sie aber dieses Vermögen bei der Fortzüchtung verloren haben und nunmehr einen mehr oder weniger ausgeprägten auxoheterotrophen Charakter aufweisen [vgl. FRIES (5), S. 1, SCHOPFER (10), S. 126]. Kürzlich haben nun WIKÉN und RICHARD (12) gezeigt, dass diese Hypothese der auxoheterotrophen Natur der Kulturhefen eine unstatthafte Verallgemeinerung enthält. Eine schweizerische Kulturweinhefe und zwar die gegen Alkohol sehr widerstandsfähige Kaltgärhefe ,,Fendant'' besitzt den Charakter eines auxo-autotrophen Organismus. Trotz ihrer ausgeprägten Auxo-Autotrophie reagiert diese Heferasse äusserst empfindlich auf sehr kleine Mengen der Wuchsstoffe (+)-Biotin und meso-Inosit, indem bereits in Anwesenheit von 0,000025γ bzw. 2,5 γ dieser Vitamine in 1 ml Nährlösung eine kleine aber deutliche, wachstumsfördernde Wirkung feststellbar war (12). In der vorliegenden Arbeit werden die Wachstumsbedingungen zwei weiterer auxo-autotropher Kulturweinhefen beschrieben. Es handelt sich dabei um die Kaltgärhefen ,,Herrliberg'' und ,,Salenegg'', welche in der Praxis zur Bereitung von Weisswein bzw. Rotwein verwendet werden (LÜTHI (7), OSTERWALDER (9)).

---

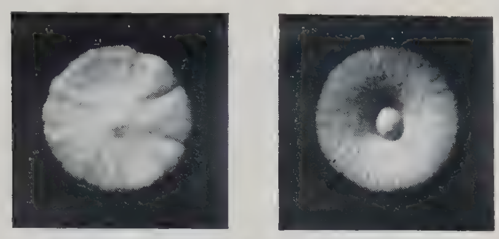
[1]) I. Mitteilung: Antonie van Leeuwenhoek **17**, 209, 1951.

Das Bild der Riesenkolonien der beiden Heferassen auf Würzegelatine geht aus Abb. 1 hervor. Die Form der Hefezellen in ungehopfter Bierwürze und Süsswein sowie in drei Nährlösungen zeigt Abb. 3. In Kulturen auf Gipsblöcken, welche mit dest. Wasser durchfeuchtet sind, bilden beide Heferassen bei 20°C. 1—4 Sporen pro Zelle (Abb. 2).

In Gärkölbchen nach Einhorn werden von den ,,Herrliberg''- und ,,Salenegg''-Hefen Fructose, Glucose und Saccharose sehr rasch unter Bildung von Kohlendioxyd angegriffen, während Galactose, Raffinose und Maltose verhältnismässig langsam dissimiliert werden, wobei die Geschwindigkeit der Gasentwicklung in der genannten Reihenfolge abnimmt. Lactose wird nicht vergoren.

METHODIK.

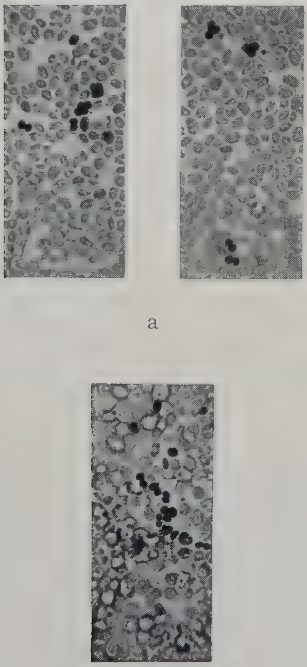
Bezüglich der bei den Untersuchungen verwendeten Methodik sowie der Zusammensetzung der verwendeten Substrate A, B^1 und C verweisen wir auf die erwähnte I. Mitteilung in dieser Zeitschrift (12). Hier sei nur erwähnt, dass als Mass des Wachstums das Absorptionsvermögen der Kulturen für Licht der Wellenlänge 660 mμ gewählt wurde. Die Messung der Lichtabsorption erfolgte mit Hilfe eines Beckman-Quarzspektrophotometers in Corex-Küvetten mit einer Schichtdicke von 10 mm, wobei die Spaltweite 0,06 mm war



a b

Abb. 1. Riesenkolonien auf Würzegelatine (siehe STELLING-DEKKER (11), S. 30–31; auf 10° Bllg verdünnte Würze; 16% Gelatine). 5 Wochen alte Kulturen. Natürliche Grösse.

a. Heferasse „Herrliberg".
b. Heferasse „Salenegg".



a

b

Abb. 2. Sporenbildung in 14 Tage alten Gipsblock-Kulturen. Färbung mit Karbolfuchsin nach Behandlung mit Chromsäure. Vergr. 425 ×.

a. Heferasse „Herrliberg".
b. Heferasse „Salenegg".

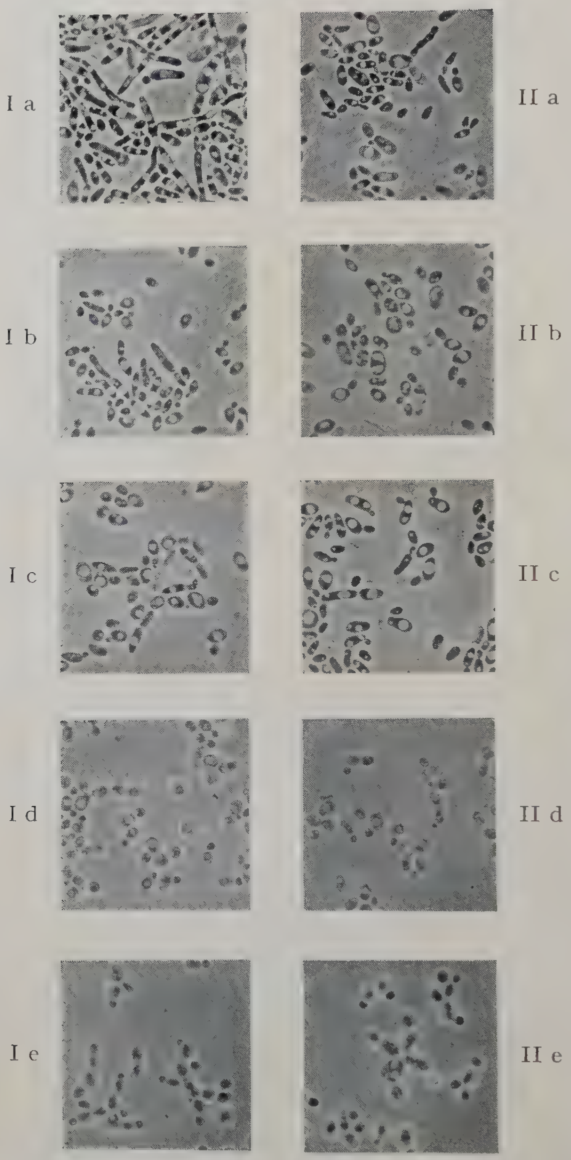


Abb. 3. Aussehen der Hefezellen in verschiedenen Nährlösungen. 48 Stunden alte Kulturen. Lebende Zellen. Phasenkontrast. Vergr. 300 ×.

I. Heferasse „Herrliberg".
II. Heferasse „Salenegg".
a. Ungehopfte Bierwürze (8° Bllg).
b. Süsswein (Verd. 1 : 1).
c. Nährlösung A.
d. Nährlösung B^1.
e. Nährlösung C.

und die nicht geimpfte Nährlösung als Vergleichslösung (Durchlässigkeit = 100 %) diente. In den Tabellen 1—10 sind die so erhaltenen Durchlässigkeitswerte (%) als Mass des Wachstums angegeben.

ERGEBNISSE UND DISKUSSION.

Heferasse „Salenegg".

In einem orientierenden Versuch wurde der Einfluss von (+)-Biotin (25 γ/1000 ml), Adermin (0,5 mg/1000 ml), Aneurin (0,5 mg/1000 ml), Pantothensäure (2,5 mg Ca-Salz/1000 ml) und meso-Inosit (25 mg/1000 ml) auf das Wachstum der „Salenegg"-Hefe in Nährlösung C, welche vitaminfreies Caseinhydrolysat als Stickstoffquelle enthält, untersucht.

TABELLE 1.

Zeit Stunden	Kein Wuchsstoffzusatz	(+)-Biotin 25 γ/ 1000 ml	Adermin 0,5 mg/ 1000 ml	Aneurin 0,5 mg/ 1000 ml	Pantothensäure 2,5 mg/ 1000 ml	meso-Inosit 25 mg/ 1000 ml
24	95,0	95,0	95,2	95,8	94,9	88,0
48	78,1	78,6	78,6	76,2	76,6	47,1
70	57,4	54,3	57,8	58,0	52,6	15,6
101	37,5	34,3	37,7	37,1	33,7	5,0
120	31,9	28,8	31,2	30,2	27,4	5,6
144	24,3	21,6	24,4	25,9	22,0	5,2
168	21,9	20,0	23,7	21,5	20,8	5,4
176	21,5	20,0	23,7	23,8	20,3	5,6

Heferasse: „Salenegg". Impfkultur: Bierwürzeschrägagar, 48 Stunden alt. Nährlösung: C. Impfung: 16.12.49. Einzelwerte.

Aus Tabelle 1 geht hervor, dass die Kulturweinhefe „Salenegg" wahrscheinlich auxo-autotropher Natur ist, indem sich ihre Zellen ohne Zusatz von Wuchsstoffen längs einer S-förmigen Kurve rasch vermehren. Allerdings stammten die in diesem Versuch als Impfmaterial verwendeten gewaschenen Zellen aus einer 48 Stunden alten Kultur auf Bierwürzeschrägagar, weshalb es nicht ausgeschlossen ist, dass die Impfzellen eine für das Wachstum im vitaminfreien Substrat C genügende Wuchsstoffmenge enthielten. Aus Tabelle 1 ist ferner ersichtlich, dass von den geprüften Wuchsstoffen nur meso-Inosit einen fördernden Einfluss auf die Vermehrung der ,,Salenegg"-Hefe ausübt.

In einem zweiten orientierenden Versuch wurde die Wirkung verschiedener Kombinationen der erwähnten Wuchsstoffe geprüft. Die Ergebnisse sind in Tabelle 2 angeführt.

TABELLE 2.

			(+)-Biotin 25 γ/1000 ml						
Zeit Stunden	Kein Wuchsstoffzusatz	(+)-Biotin 25 γ/1000 ml	Adermin 0,5 mg/1000 ml	Aneurin 0,5 mg/1000 ml	Pantothensäure 25 mg/1000 ml	meso-Inosit 25 mg/1000 ml	Panthothensäure 2,5 mg/1000 ml		
							Adermin 0,5 mg/1000 ml	Aneurin 0,5 mg/1000 ml	meso-Inosit 25 mg/1000 ml
24	85,9	82,6	84,2	82,7	81,7	68,9	83,0	78,1	66,8
48	58,7	60,0	58,8	60,1	55,0	23,3	54,0	58,5	16,3
73	41,0	43,3	40,7	44,0	40,0	8,2	39,0	40,6	11,2
96	34,3	33,5	34,0	39,3	30,0	6,5	28,2	31,0	6,3
120	22,1	28,9	26,8	27,4	25,5	6,3	22,4	23,7	6,8
168	22,0	20,5	22,5	22,5	18,6	6,3	18,5	19,0	6,0
192	17,6	18,0	17,0	14,4	12,3	6,8	18,0	19,0	5,9

Heferasse: ,,Salenegg". Impfkultur: Bierwürzeschrägagar, 70 Stunden alt. Nährlösung: C. Impfung: 9.1.50. Einzelwerte.

Es ist ersichtlich, dass nur die Kombinationen, welche meso-Inosit enthalten, eine Förderung des Wachstums der ,,Salenegg"-Hefe in Nährlösung C bewirken. Dabei ist die Wirkung der Kombination von meso-Inosit und (+)-Biotin gleich gross wie der Einfluss des Gemisches vom ersten Wuchsstoff mit (+)-Biotin und Pantothensäure.

Da im Falle der ,,Salenegg"-Hefe dem meso-Inosit somit eine wachstumsfördernde Wirkung zugeschrieben werden muss und ferner in unseren Untersuchungen über die Wachstumsbedingungen der auxo-autotrophen ,,Fendant"-Hefe nicht nur in Anwesenheit des meso-Inosits, sondern auch nach Zusatz des (+)-Biotins ein kleiner aber deutlicher, fördernder Einfluss feststellbar war (12), setzten wir die Versuche mit diesen beiden Vitaminen fort. Dabei wurde zuerst die Wirkung einer Kombination von (+)-Biotin und meso-Inosit (25γ bzw. 25 mg/1000 ml Substrat) mit dem Einfluss gleicher Mengen der einzelnen Wuchsstoffe auf das Wachstum der „Salenegg"-Hefe in den Nährlösungen B¹ (Ammoniumsulfat als Stickstoffquelle) und C (Caseinhydrolysat als Stickstoffquelle) verglichen. Die Ergebnisse gehen aus Tabelle 3 hervor.

TABELLE 3.

Zeit Stunden	Nährlösung B^1				Nährlösung C			
	Kein Wuchsstoffzusatz	(+)-Biotin 25 γ/1000 ml	meso-Inosit 25 mg/1000 ml	(+)-Biotin 25 γ 1000 ml meso-Inosit 25 mg/1000 ml	Kein Wuchsstoffzusatz	(+)-Biotin 25 γ/1000 ml	meso-Inosit 25 mg/1000 ml	(+)-Biotin 25 γ 1000 ml meso-Inosit 25 mg/1000 ml
24	92,6	90,0	82,3	82,1	86,9	86,9	75,6	77,9
48	61,2	57,1	25,2	24,0	56,5	58,2	21,8	20,7
72	37,2	33,7	10,5	10,1	37,5	37,9	7,5	8,4
96	24,7	24,5	7,7	7,6	24,9	21,8	7,3	7,9
120	17,3	16,9	7,0	7,1	19,4	18,6	6,6	7,6
168	12,6	12,3	6,7	6,0	15,6	16,1	6,2	6,5
240	11,6	—	—	—	14,2	—	—	—

Heferasse: „Salenegg".
Impfkulturen: In den B¹-Reihen Nährlösung B¹, 72 Stunden alt; in den C-Reihen Nährlösung C, 72 Stunden alt.
Impfung: 12.2.51. 5 Parallelen.

Es ist ersichtlich, dass sich die Heferasse „Salenegg" auch in diesem Versuch ohne irgendwelchen Zusatz von Wuchsstoffen längs einer normalen S-Kurve rasch vermehrt. Da als Impfmaterial Zellen dienten, welche durch zahlreiche aufeinanderfolgende Passagen in den erwähnten wuchsstofffreien Substraten B¹ und C gewonnen waren, ist die auxo-autotrophe Natur der „Salenegg"-Hefe eindeutig bewiesen. Tabelle 3 zeigt ferner, dass (+)-Biotin (25γ/1000 ml) weder auf Ammoniumsulfat (B¹) noch auf Aminosäuren (C) als Stickstoffquelle eine Wachstumsförderung bewirkt. Dagegen übt meso-Inosit (25 mg/1000 ml) einen sehr starken fördernden Einfluss auf die Vermehrung der Hefezellen in beiden Nährlösungen aus. In den Reihen, welche ein Gemisch von (+)-Biotin und meso-Inosit enthalten, ist das Wachstum gleich gross wie in der entsprechenden

Reihe mit Zusatz von meso-Inosit allein. Das (+)-Biotin vermag somit den günstigen Effekt des meso-Inosits auf die Vermehrung der auxo-autotrophen „Salenegg"-Hefe nicht zu erhöhen.

Schliesslich wurden in den Nährlösungen B^1 und C Versuche durchgeführt, teils um die kleinste wachstumsfördernde Konzentration des meso-Inosits zu bestimmen, teils um abzuklären, ob bei Konzentrationen von (+)-Biotin, welche grösser als 25γ je 1000 ml Substrat sind, eine günstige Wirkung auf die Vermehrung der „Salenegg"-Hefe auftritt. Die Ergebnisse einiger typischen Versuche sind in den Tabellen 4—6 angeführt. Es ist ersichtlich, dass meso-Inosit bereits in einer Menge von 2,5 mg je 1000 ml der Nährlösungen B^1 und C (2,5 γ/1 ml) einen fördernden Einfluss auf das Wachstum ausübt (Tabelle 4). Diese Wirkung erreicht in Anwesenheit von 25 mg des Vitamins je 1000 ml Nährlösung ihr Maximum, indem bei dieser Konzentration das Wachstum gleich gross wie in Gegenwart der zehnfachen Wuchsstoffmenge ist (Tabellen 5 und 6). Die Versuchsergebnisse zeigen ferner eindeutig, dass (+)-Biotin weder auf Ammoniumsulfat (B^1) noch auf Aminosäuren (C) als Stickstoffquelle einen Wuchsstoffeffekt ausübt und zwar nicht einmal, wenn die Konzentration des Vitamins auf 100 bzw. 250 γ je 1000 ml Nährlösung erhöht wird. Ausserdem wird bestätigt, dass die Wirkung einer Kombination der beiden Wuchsstoffe gleich

TABELLE 4.

Zeit Stunden	Nährlösung B^1				Nährlösung C			
	Kein Wuchsstoffzusatz	meso-Inosit mg/1000 ml			Kein Wuchsstoffzusatz	meso-Inosit mg/1000 ml		
		0,25	2,5	25		0,25	2,5	25
24	77,9	73,3	61,8	61,2	74,6	69,9	58,2	57,0
48	43,4	42,4	21,2	12,6	42,6	38,9	16,4	10,2
72	30,5	29,4	13,6	7,2	31,0	27,2	10,2	7,3
96	21,2	21,6	10,7	6,5	20,6	18,4	9,4	6,8
120	15,5	15,2	10,5	6,3	17,0	15,9	9,2	6,4
144	12,2	14,2	9,4	6,5	14,8	13,9	9,3	6,2

Heferasse: „Salenegg".
Impfkulturen: In den B^1-Reihen Nährlösung B^1, 72 Stunden alt; in den C-Reihen Nährlösung C, 72 Stunden alt.
Impfung: 2.3.51. 5 Parallelen.

gross wie der Einfluss des meso-Inosits allein ist (Tabellen 5 und 6).

TABELLE 5.

Zeit Stunden	Kein Wuchsstoffzusatz	(+)-Biotin γ/1000 ml			meso-Inosit mg/1000 ml		meso-Inosit 25 mg/1000 ml (+)-Biotin γ/1000 ml	
		25	100	250	25	250	25	250
24	66,4	82,6	80,2	78,5	63,5	64,3	65,2	61,7
48	39,4	50,6	48,5	48,2	19,6	19,4	22,7	21,2
72	29,6	34,7	35,1	32,2	7,8	7,8	7,9	10,1
96	20,8	21,8	22,3	22,1	7,9	6,7	7,2	6,9
120	15,8	17,2	19,1	16,4	6,1	6,3	6,5	6,6
168	12,7	12,0	13,2	12,9	6,6	6,4	6,4	6,7

Heferasse: „Salenegg". Impfkultur: Nährlösung B^1, 96 Stunden alt. Nährlösung: B^1. Impfung: 12.3.51. 5 Parallelen.

TABELLE 6.

Zeit Stunden	Kein Wuchsstoffzusatz	(+)-Biotin γ/1000 ml			meso-Inosit mg/1000 ml		meso-Inosit 25 mg/1000 ml (+)-Biotin γ/1000 ml	
		25	100	250	25	250	25	250
24	80,7	79,7	80,5	80,0	72,8	72,8	74,2	73,2
48	52,3	52,4	51,3	51,0	22,2	19,6	19,2	19,3
72	32,4	30,7	30,2	30,4	10,1	9,1	8,9	8,2
96	19,4	20,1	20,5	18,9	7,3	7.5	7,2	7,4
120	16,8	16,7	15,9	16,0	7,1	7,0	7,3	7,3
168	14,8	13,9	13,6	14,5	6,8	6,6	6,0	6,5

Heferasse: „Salenegg". Impfkultur: Nährlösung C, 96 Stunden alt. Nährlösung: C. Impfung: 12.3.51. 5 Parallelen.

Heferasse „Herrliberg".

Die Ergebnisse einiger Versuche mit der „Herrliberg"-Hefe sind in den Tabellen 7—10 zusammengestellt. Aus diesen geht eindeutig hervor, dass sich diese Heferasse bezüglich des Wuchsstoffbedarfes wie die „Salenegg"-Hefe verhält. Sie zeigt ebenfalls eine ausgesprochene Auxo-Autotrophie, indem in sämtlichen Versuchen in den

Kulturen ohne Wuchsstoffzusatz eine rasche Zellvermehrung längs einer normalen, S-förmig ansteigenden Kurve stattfand. Die Prüfung des Einflusses der teils einzeln, teils in Kombinationen zugesetzten Vitamine (+)-Biotin, Adermin, Aneurin, Pantothensäure und meso-Inosit zeigte, wie aus den Tabellen 7 und 8 ersichtlich ist,

TABELLE 7.

Zeit Stunden	Kein Wuchsstoffzusatz	(+)-Biotin 25 γ/1000 ml	Adermin 0,5 mg/1000 ml	Aneurin 0,5 mg/1000 ml	Pantothensäure 2,5 mg/1000 ml	meso-Inosit 25 mg/1000 ml
24	96,7	96,6	97,1	97,0	96,8	88,8
48	80,5	81,4	82,2	82,6	80,5	58,0
70	62,3	66,4	66,5	64,7	64,2	26,7
101	44,0	45,0	46,6	46,9	46,2	7,8
120	41,0	40,7	41,5	44,2	41,9	6,6
144	34,6	37,1	38,6	36,2	39,2	5,8
168	33,8	35,9	38,4	34,4	35,8	5,3
176	33,9	35,5	38,0	35,0	35,0	5,8

Heferasse: „Herrliberg''. Impfkultur: Bierwürzeschrägagar, 48 Stunden alt. Nährlösung: C. Impfung: 16.12.49. Einzelwerte.

TABELLE 8.

Zeit Stunden	Kein Wuchsstoffzusatz	(+)-Biotin 25 γ/1000 ml	(+)-Biotin 25 γ/1000 ml						
			Adermin 0,5 mg/1000 ml	Aneurin 0,5 mg/1000 ml	Pantothensäure 2,5 mg/1000 ml	meso-Isonit 25 mg/1000 ml	Pantothensäure 2,5 mg/1000 ml		
							Adermin 0,5 mg/1000 ml	Aneurin 0,5 mg/1000 ml	meso-Inosit 25 mg/1000 ml
24	86,8	87,0	87,3	87,8	86,3	66,8	86,0	87,6	66,6
48	65,0	65,8	64,0	64,0	63,3	27,3	64,5	64,0	26,2
73	46,3	49,4	53,9	52,6	47,2	9,0	49,5	51,0	7,0
96	43,7	41,2	43,6	42,9	40,0	7,5	37,9	41,3	5,6
120	40,9	36,5	36,7	34,5	35,3	6,8	32,3	35,3	5,3
168	28,0	28,0	27,9	27,5	26,8	6,3	30,6	27,0	6,0
192	30,0	27,4	26,0	23,9	24,8	5,8	24,7	27,0	5,8

Heferasse: „Herrliberg''. Impfkultur: Bierwürzeschrägagar, 70 Stunden alt. Nährlösung: C. Impfung: 9.1.50. Einzelwerte.

dass im Falle der „Herrliberg"-Hefe nur dem letzten Wuchsstoff eine wachstumsfördernde Wirkung zugeschrieben werden kann. Die Auxo-Autotrophie der „Herrliberg"-Hefe sowie der fördernde Ein-

TABELLE 9.

	Nährlösung B^1				Nährlösung C			
Zeit Stun-	Kein Wuchsstoffzusatz	(+)-Biotin 25 γ 1000 ml	meso-Inosit 25 mg/1000 ml	(+)-Biotin 25 γ/1000 ml meso-Inosit 25 mg/1000 ml	Kein Wuchsstoffzusatz	(+)-Biotin 25 γ/1000 ml	meso-Inosit 25 mg/1000 ml	(+)-Biotin 25 γ/1000 ml meso-Inosit 25 mg/1000 ml
24	90,2	90,1	85,1	84,6	93,2	92,2	90,1	89,6
48	59,6	57,0	32,2	27,5	61,7	59,5	32,3	31,5
72	37,2	42,1	11,6	10,8	36,4	36,4	10,6	9,2
96	26,2	29,2	6,9	6,6	23,6	24,2	6,4	6,3
120	18,3	22,1	6,3	5,7	18,5	17,6	6,4	6,1
168	13,1	14,3	6,1	5,6	13,7	13,7	5,9	6,3
240	12,0	—	—	—	13,1	—	—	—

Heferasse: „Herrliberg".
Impfkulturen: In den B^1-Reihen Nährlösung B^1, 72 Stunden alt; in den C-Reihen Nährlösung C, 72 Stunden alt.
Impfung: 12.2.51. 5 Parallelen.

TABELLE 10.

	Nährlösung B^1				Nährlösung C			
Zeit-Stunden	Kein Wuchsstoffzusatz	meso-Inosit mg/1000 ml			Kein Wuchsstoffzusatz	meso-Inosit mg/1000 ml		
		0,25	2,5	25		0,25	2,5	25
24	77,1	74,1	65,4	64,9	65,3	62,6	51,0	51,2
48	43,1	40,9	23,0	19,3	27,1	25,0	14,8	9,5
72	33,2	30,1	14,9	9,6	20,1	19,5	11,2	5,8
96	23,8	24,1	11,5	6,3	14,5	15,1	9,2	5,8
120	16,2	20,0	9,9	5,8	12,5	11,9	9,6	5,9
144	13,5	15,4	9,7	6,0	11,4	12,1	9,7	5,8

Heferasse: „Herrliberg".
Impfkulturen: In den B^1-Reihen Nährlösung B^1, 72 Stunden alt; in den C-Reihen Nährlösung C, 72 Stunden alt.
Impfung: 2.3.51. 5 Parallelen.

fluss des meso-Inosits auf ihr Wachstum in den Nährlösungen B¹ (mit Ammoniumsulfat) und C (mit Caseinhydrolysat) werden durch die in den Tabellen 9 und 10 angeführten Ergebnisse erläutert. Bei diesen Versuchen dienten als Impfmaterial Zellen, welche durch zahlreiche Passagen in den wuchsstofffreien Lösungen B¹ und C gewonnen waren. Die Zahlen der Tabelle 9 bestätigen, dass meso-Inosit eine kräftige Wachstumsförderung bewirkt, während (+)-Biotin allein keinen Einfluss hat und auch nicht die günstige Wirkung des meso-Inosits zu erhöhen vermag. Tabelle 10 zeigt, dass die „Herrliberg"-Hefe gleich der obenerwähnten „Salenegg"-Hefe sowie der früher beschriebenen „Fendant"-Hefe (12) bereits auf die Anwesenheit von 2,5 mg meso-Inosit je 1000 ml Nährlösung reagiert.

Schliesslich sei erwähnt, dass mit den „Salenegg"- und „Herrliberg"-Hefen Hunderte von aufeinanderfolgenden Passagen in den Nährlösungen B¹ und C ohne Zusatz von Wuchsstoffen mit gutem Erfolg durchgeführt wurden.

Diese Arbeit ist ein Teil der Untersuchungen über die Physiologie der Weinhefen, welche von der Abteilung für Landwirtschaft des Eidg. Volkswirtschaftsdepartements aus dem Weinbaufonds unterstützt werden. Den zuständigen Behörden sind wir zu grossem Dank verpflichtet.

Die „Herrliberg"- und „Salenegg"-Hefen erhielten wir von der Eidg. Versuchsanstalt für Obst-, Wein- und Gartenbau, Wädenswil. Herrn Professor Dr F. Kobel sowie Herrn Dr H. Lüthi möchten wir an dieser Stelle für ihre Liebenswürdigkeit herzlich danken.

Fräulein L. Bendel danken wir bestens für sorgfältige Assistenz.

Summary.

In a preceding paper (12) the authors described some results obtained in studying the growth of the Fendant yeast, a selected Swiss tame yeast used at low temperatures for production of (white) wines of high alcoholic content. It was shown conclusively that this culture yeast is an auxo-autotrophic strain able to grow in a synthetic medium containing only glucose and mineral salts including ammonium sulfate. The addition of (+)-biotin in amounts greater than 0.0025—0.025 γ per 1000 ml or meso-inositol in quantities greater than 0.25—2.5 mg per 1000 ml has only a small beneficial action upon growth. The authors concluded that the theory of the auxo-heterotrophic nature of culture yeasts involves an inadmissible generalization.

The present paper reports the results obtained in investigating two more Swiss culture yeasts employed in preparation of wine at low temperatures, namely the sporogenous races Salenegg (red wine) and Herrliberg (white wine). The growth was examined in nutrient solutions containing glucose, mineral salts, and ammonium sulfate (substrate B[1]) or casein hydrolysate (substrate C) as a source of nitrogen. The absorption of light in the Beckman photoelectric quartz spectrophotometer at the wave length 660 mμ was used as a measure of growth. The photometer readings in percentage transmission are given in the tables showing yeast growth as a function of time.

The growth experiments show that the culture yeasts Salenegg and Herrliberg are autotrophic for growth factors, normal growth being obtained in the above nutrient solutions without addition of vitamins.

Of the growth factors tested (+)-biotin, pyridoxine (adermin), thiamin (aneurine), and pantothenic acid, added singly and in various combinations, have no stimulating action upon growth of the tame yeasts Salenegg and Herrliberg, whereas addition of meso-inositol in amounts greater than 0.25—2.5 mg per 1000 ml of nutrient solution produces a marked increase in growth. Maximum growth is attained in presence of 25 mg of meso-inositol per 1000 ml of substrate, the yields remaining the same on addition of greater amounts of the vitamin (250 mg/1000 ml). The stimulating effect of meso-inositol is not increased by (+)-biotin, added singly or in combination with pantothenic acid.

During current investigations in this laboratory the culture yeasts Salenegg and Herrliberg were successfully subcultured about 150 times in the chemically defined nutrient solutions mentioned above without an exogenous supply of vitamins.

The results dealt with in the present article confirm the view proposed by the authors (12) that it is incorrect to generalize the theory of auxo-heterotrophy of tame yeasts.

Literatur.

1. L. Atkin, P. P. Gray, W. Moses and M. Feinstein, European Brewery Convention, Congr. Lucerne, I, 96, 1949. – 2. P. R. Burkholder, Amer. J. Bot. **30**, 206, 1943. – 3. A. M. Copping, Biochem. J. **23**, 1050, 1929. – 4. W. B. Emery, N. McLeod and F. A. Robinson, Biochem. J. **40**, 426, 1946. – 5. N. Fries, Symb. Bot. Upsal. III : **2**, 1938. – 6. L. H. Leonian

and V. G. Lilly, Amer. J. Bot. **29,** 459, 1942. – 7. H. Lüthi, Mitteil. Eidg. Versuchsanstalt Wädenswil, Flugschr. **12,** 1947. – 8. G. Menzinsky, Ark. f. Kemi (Stockholm) **2** : **1,** 1950. – 9. A. Osterwalder, Zentralbl. f. Bakt. II, **90,** 226, 1934. – 10. W. H. Schopfer, Plants and Vitamins. Waltham, Mass. 1949. – 11. N. M. Stelling-Dekker, Die sporogenen Hefen. Amsterdam 1931. – 12. T. Wikén und O. Richard, Antonie van Leeuwenhoek **17,** 209, 1951. – 13. R. J. Williams, R. E. Eakin and E. E. Snell, J. Amer. Chem. Soc. **62,** 1204, 1940. – 14. R. J. Williams and E. Rohrman, J. Amer. Chem. Soc. **58,** 695, 1936.

(Netherlands Experiment Station for the Utilization of Straw, Groningen).

ON THE UTILIZATION OF WASTE LIQUORS FROM THE DIGESTION OF STRAW WITH MONO-SULPHITE

by

H. F. BANNINK and **F. M. MULLER**

(Received August 15, 1951).

§ 1. INTRODUCTION.

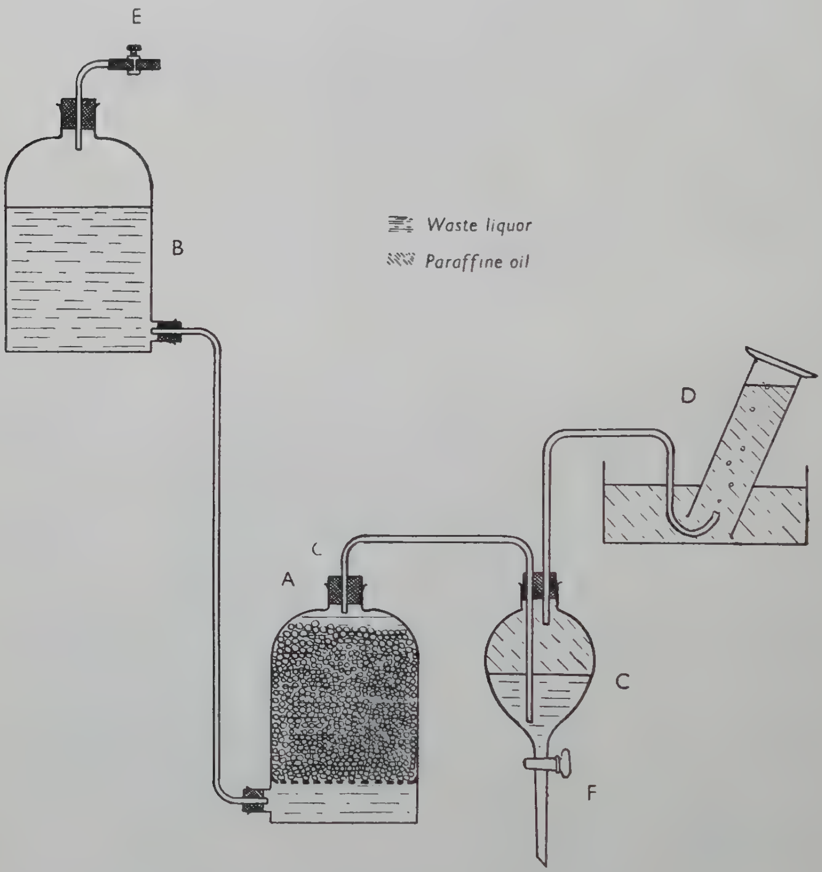
Among the processes used in the Netherlands for the manufacture of cardboard, paper pulp, etc. from straw, digestion with sodium monosulphite plays an increasingly important rôle. In a former paper (3) attention was drawn to the noxious effect which the sulphite in the waste liquor of this digestion may have on methane production from this waste and the sensitivity to sulphite of methane fermentation of acetate was ascertained. At a pH of 6.0—6.5 a concentration of 0.003 to 0.01 % of Na_2SO_3 appeared to cause a perceptible inhibition of the latter process. At the end of this paper it was proposed to try to remove the sulphite from the waste liquor with the aid of sulphate-reducing bacteria which are known to convert all kinds of inorganic sulphur compounds to hydrogen sulphide, the latter compound being relatively harmless for methane bacteria (see (1)).

The present paper deals with this conversion of sulphite in the waste liquor and with the subsequent removal of the hydrogen sulphide formed, in order to obtain a less offensive effluent.

§ 2. APPARATUS EMPLOYED.

The essential part of the apparatus employed (see figure) is a 1 l decanting bottle A with a piece of monel wire gauze right above the lower orifice, supported by strips of wood veneer (not shown).

On this gauze matted glass beads are piled, filling the bottle up to a short distance from the neck. The beads serve as a support for the sulphate-reducing bacteria and increase the area of contact with the sulphite-containing waste liquor [1]) flowing from storage bottle B. The rate of flow can be controlled with the screw clamp E on the air entrance to B.



The liquor ascending in A is subsequently conducted by a U tube into separation funnel C and is collected here under paraffine oil. Any gas carried over with the liquor will leave C together with the replaced paraffine oil and collect in calibrated cylinder D where the volume can be read off and samples taken for analysis. The liquor can be withdrawn from C through stopcock F.

---

[1]) The importance of a support for the conversion of small concentrations of constituents of the medium is outlined by ZoBell (7).

The liquor used in the experiments was obtained from the digestion of straw with about 8 % Na-monosulphite and 2 % Na-hydroxide, calculated on straw. The liquor separated from the pulp after digestion was about ten times more concentrated then a technical waste liquor and thus was diluted tenfold with water before use. The whole batch was sterilized in 5 l portions so that all experiments could be carried out with the same liquor.

Bottle A was inoculated with a 250 ml enrichment culture of sulphate-reducing bacteria. Later on a second apparatus was set up and inoculated with sludge from the settling pond of a strawboard mill.

§ 3. ANALYTICAL METHODS.

The search for suitable analytical methods took up a lot of time. The usual methods for the determination of sulphite and sulphide were not fit for the liquor under consideration because the organic constituents interfered.

The main point was to know the total S content of the liquor, the part of it present in the form of sulphite, whether there was any sulphite left after passage of bottle A and the amount of H_2S produced by the bacteria.

Originally total S was determined by fusion of the evaporation residue of the liquor with KNO_3 and KOH (5). However, in order to avoid the time-consuming evaporation, a different method was used later on, based upon oxidation in the liquor as such with a mixture of HNO_3 and $HClO_4$ (4), which was checked against the former method with satisfactory results.

Sulphite was determined by distilling the acidified liquor, followed by iodine titration, and by direct titration with iodine. When checking these methods with strawboard waste liquor to which some sulphite had been added, direct titration appeared to be preferable. If the raw liquor also contains H_2S this should be precipitated first (e.g. by adding Zn-acetate followed by filtration).

Most difficulties were encountered in the determination of H_2S in the effluent of bottle A. Direct titration with iodine (after dilution of the rather dark liquor so that the colour change could be observed) yielded much too high figures. Then acid distillation, followed by iodine titration of the distillate was tried, but the figures were still too high. Various modifications of the distillation procedure were tried, e.g. passing CO_2 through the flask before and after distillation, distillation with phosphoric or hydrochloric acid, but none of these yielded satisfactory results. By precipitating H_2S with Pb-acetate, followed by distillation with HCl it was found that compounds were carried over which also used up iodine.

For this reason it was tried to collect the H_2S distilled in ammoniacal hydrogen peroxide solution, followed by gravimetric determination of the H_2SO_4 (formed through oxidation of the H_2S) as $BaSO_4$. The figures so obtained were a little more than half those from distillation + iodine titration and were in accordance with the sulphur balance (S in H_2S = total S in effluent — total S in distillation residue).

In order to decrease the time necessary for the determination various other methods were tried, a.o. one based on the statement of BOLDESCHWIELER (2) that all lead salts, with the exception of PbS, are soluble in boiling ammonium acetate solution. However, difficulties with the filtration rendered this method unsuitable for the present case. Also a titration under vacuum (6) did not meet with success. Direct titration with 0.1 N iodine in a microburette yielded somewhat too high figures which, however, may be considered suitable for orientation. Finally a differential titration also yielded satisfactory results: an excess of acidified iodine solution was added to the liquor and titrated back. In a different portion the H_2S was precipitated with Zn-acetate, and the liquor titrated after filtration, the H_2S content being calculated from the difference between the two titrations.

In conclusion it may be stated that the determination of the H_2S by distillation and collection in ammoniacal H_2O_2 solution was the most reliable one. The distilling apparatus was flushed before and after distillation with hydrogen, in order to remove all oxygen and to carry over all H_2S into the peroxide solution respectively.

§ 4. HYDROGEN SULPHIDE PRODUCTION IN THE WASTE LIQUOR.

The liquor employed had the following composition:

dry matter	0.99 %
loss on ignition	0.73 %
pH	9.0
total sulphur	550 mg/l
sulfite (calc. as S)	32 ,,

Thus, total sulphur appeared to be rather low and the part of it present as sulphite much lower still. Thus, the far greater part of the sulphur was present in organic combination.

The liquor percolated through the apparatus with a speed of about 1 l per 24 h. The pH of the effluent was about 6.5. A higher speed of the liquor was always accompanied by a decrease in H_2S production, but a lower speed did not yield an increase. After adaptation of the bacteria the H_2S content of the effluent varied for a long time between 100 and 140 mg/l. A regular small addition of nitrogen in the form of ammonium chloride to the influent did not increase the H_2S production. The effluent never contained any sulphite.

These figures show clearly that the sulphite present was completely reduced to H_2S and that between 18 and 27 % of the total sulphur was converted to H_2S.

As a control the total sulphur in the effluent was determined a few times and always found to be about 460 mg/l, i.e. 90 mg/l below that of the influent. This difference can hardly be due to accumulation of sulphur in bottle A, as it was found, at different points of time wide apart [1]. However, it seemed possible that H_2S had dissolved in the paraffine oil and had escaped with this liquid from the funnel into the dish at D.

In order to test this possibility a calibrated cylinder was partially filled with pure H_2S and put upside down in a dish with paraffine oil at 30° C. in the same manner as in the experiments and the level of the oil in the cylinder noted at different intervals. It appeared that in 72 hours 42 ml of H_2S disappeared from a cylinder with a cross section of 28 cm². Thus, a possibility of escape of H_2S with the oil did exist.

In a following experiment a suspension of iron oxide hydrate in water (sample A, see § 5) was brought in the separation funnel below the oil before the liquor was admitted, in order to fix all H_2S. The total S content amounted now to 476 mg/l which was still 74 mg/l below that of the influent of bottle A.

Another possibility of escape of H_2S was with the gas produced in bottle A (see below). This was checked by connecting two gas washing bottles containing a lead acetate solution to the gas outlet at D and gravimetric determination of the PbS formed. However, passage of 970 ml waste liquor through the apparatus yielded only 10 mg of PbS, equivalent to 1.3 mg S. This shows that practically no H_2S disappeared from the system with the gas.

---

[1] In the beginning the monel gauze will have fixed some H_2S, while moreover, some H_2S will have been precipitated by the iron in the liquor as FeS. However, the iron content of the liquor was 6.5 mg/l which can fix only 3.7 mg/l of H_2S.

About two months after the start of the experiment gas bubbles appeared among the beads in bottle A which gas was collected in the calibrated cylinder at D. The yield amounted to 500—700 ml per l of influent. The gas consisted of methane and carbon dioxide in a ratio of about 2 : 1:

Date	CH_4	CO_2	CH_4/CO_2 ratio
10/18 '49	60.4 %	27.0 %	2.2
10/19 '49	56.7 %	30.4 %	1.8
10/24 '49	58.1 %	29.5 %	1.9
11/18 '49	54.0 %	32.2 %	1.7
5/2 '50	67.1 %	22.6 %	3.0
			Av. 2.1

The ignition loss of the effluent varied between 0.60—0.66 %, i.e. on the average 0.1 % below that of the influent. If it is assumed that the ignition loss has the composition of a hexose ($C_6H_{12}O_6$) and that half of the CO_2 produced remained dissolved in the liquor, 1 g/l (0.1 %) will yield about 550 ml of gas which is in satisfactory agreement with the observed yields. The amount of organic matter oxidized by the sulphate-reducing bacteria is negligibly small.

In order to find out whether still more H_2S could be produced from the liquor, the effluent was treated with iron shavings which reduced the H_2S content to 20 mg/l. The effluent was then passed through the apparatus a second time, but no more H_2S was produced from it. Evidently the sulphur compounds remaining in the effluent could not be reduced by the bacteria present in the apparatus.

Finally another 1 l portion of waste liquor was passed through the apparatus in the course of 22½ hours and all determinations with in- and effluent were repeated.

Gasproduction amounted to 610 ml. The analytical results were as follows:

	Influent	Effluent
pH (potentiometric)	9.2	6.9
dry matter (%)	1.14	0.89
ignition residue (%)	0.35	0.21
,, loss (%)	0.79	0.68
total S (mg/l)	554	461
sulphite S (mg/l)	32	nil
sulphide S (mg/l)	nil	95 [1]) 91 [2]) 99 [3])
S in distillation residue (mg/l)	—	342

The three determinations of H_2S check satisfactorily. Total S in the distillation residue + H_2S (1st determination) should be equal to total S in effluent (461 mg/l); with a view to the small amounts involved the difference is acceptable. Also in this experiment total sulphur in the effluent was about 90 mg/l below that of the influent.

At the end of the investigation the whole contents of bottle A were brought on a Büchner funnel, the adhering liquid filtered off and the residue, consisting of the beads, the monel gauze with its support and the adhering organic matter (which had a deep black colour) washed with distilled water. The whole mass was then put into a flask and covered with boiled dilute hydrochloric acid. Thereupon the black colour disappeared and a large part of the adhering matter went into solution. However, the liquid thus obtained did not contain any H_2S or S. Since in the aggregate about 250 l of liquor had been passed through the bottle, this should have contained 250 × 90 mg = about 22.5 g of sulphur if the sulphur loss were due to sulphur retention in the bottle. If this sulphur had been fixed in the form of an insoluble sulphide it should have shown up in this test.

Therefore, no satisfactory explanation can be given for the shortage of about 90 mg/l of total S in the effluent of bottle A.

§ 5. REMOVAL OF THE HYDROGEN SULPHIDE FROM THE TREATED SULPHITE WASTE LIQUOR.

It was tried to remove the H_2S from the effluent with iron oxide hydrate, four samples of which were kindly put at our disposal by Messrs. "N.V. Intertransport" from Rotterdam. The composition of these samples was as follows:

---

[1]) Distillation into ammoniacal H_2O_2 solution and precipitation as $BaSO_4$.

[2]) Direct titration with 0.1 N I_2 in microburette.

[3]) Differential titration.

Sample	H_2O %	Fe_2O_3 %	insol. HCl %	Fe_2O_3 % of dry matter
A	62.8	7.6	0.5	20.4
B	77.2	14.3	1.5	62.6
C	29.1	39.6	20.8	55.9
D	16.2	41.8	29.6	49.9

For these experiments the effluent was tapped from the funnel into 300 ml stoppered bottles which were filled completely. Various amounts of the four samples were added to these bottles and distributed throughout the liquid by rolling the bottles. After ½ hour of settling the H_2S content of the supernatant liquid was determined in portions of liquid filtered through filter paper. Determinations after 1 and 7 hours and after centrifuging yielded the same results as after ½ hour of settling. The results are given below.

Amount of sample per bottle, g	Sample			
	A	B	C	D
	H_2S content, mg/l			
1	46	21	57	62
2	30	33	50	57
4	23	18	46	50
½		23		

Approximately the same results were obtained when the experiment was repeated after considerable time with 1 g of each of the samples per bottle:

A	B	C	D
35	18	37	77

Notwithstanding the fact that sample B had the lowest Fe_2O_3 content but one, it yielded by far the best results. This sample consisted of sludge from the de-ironing of ground water.

The treatment rendered the liquor quite black which also remained this way after filtration, presumably through very small particles of iron sulphide which did not settle. This would explain the small residual amount of H_2S in the treated liquor. At any rate 0.5 g of sample B was sufficient to remove the H_2S from 300 ml of liquor as much as possible. This amounts to 1.7 kg per m^3 of liquor.

§ 6. DISCUSSION.

The results show that it is possible to convert about 20 % of the total sulphur in a monosulphite waste liquor, including all the sulphite, to hydrogen sulphide and to remove the latter from the waste with some cheap form of iron oxide hydrate. The waste thus treated is amenable to methane fermentation.

In order to obtain an insight into the practical possibilities of the method the present small scale investigation will have to be supplemented by trials on a semi-technical scale, using materials like blast furnace slag or coke as a support for the sulphate-reducing bacteria. However, it may be expected that the tank volume necessary for a conversion of all the biologically reducible sulphur compounds of the waste liquor to H_2S will not have to be larger than the daily volume of this liquor. Also in such a large unit methane fermentation will probably occur in the higher regions of the tank where sulphite is absent (the small amount of sulphite will be the first sulphur compound which the sulphate-reducing bacteria will attack). Therefore, when detoxication of the liquor with a view to subsequent methane production is the only purpose of the treatment, a much smaller tank volume will probably suffice.

As the effluent of bottle A still contained about 85 % of the ignition loss of the influent, methane fermentation in this bottle evidently did not run to completion (this process reduces the ignition loss in similar waste liquors to about 50 %). An efficient technical installation might consist of a small sulphite-reducing tank (of e.g. $\frac{1}{4}$ of the daily volume) and a large methane fermentation tank (of e.g. twice the daily volume). In the latter the remainder of the biologically reducible sulphur compounds will then be converted to H_2S simultaneously with the methane fermentation. If desired the H_2S can be removed from the final effluent by the addition of iron oxide hydrate in a small mixing tank, followed by a settling tank with a detention period of e.g. a couple of hours.

Summary.

1. It is possible to convert the free sulphite in the waste liquor of a monosulphite digestion of straw in a short time to hydrogen sulphide by percolating the liquor under anaerobic conditions, through a solid substrate to which sulphate reducing bacteria are attached.
2. Besides the sulphite, part of the other sulphur compounds in the liquor are converted to H_2S (about 1/5 of the total S).
3. A methane fermentation can develop in the liquor treated in this manner.
4. In a liquor with 550 mg/l total sulphur only 460 mg/l were found after the treatment; for this loss of sulphur no satisfactory explanation was found.
5. It is possible to remove the H_2S from the treated liquor with the aid of iron oxide hydrate; a suitable material for this purpose is the de-ironing sludge of ground water.
6. For the determination of H_2S in the treated liquor the most reliable method appeared to be distilling of the acidified liquor into an ammonical solution of hydrogen peroxide and precipitation of the sulphate ion formed as Ba-sulphate.
7. The possibility of a combination of sulphite reduction, methane fermentation and sulphide removal on a technical scale is discussed.

Bibliography.

1. H. F. Bannink and F. M. Muller, Antonie van Leeuwenhoek **17**, 171, 1951. – 2. E. L. Boldeschwieler, Anal. Chem. **6**, 402, 1934. – 3. M. A. Noordam-Goedewaagen, A. Manten and F. M. Muller, Antonie van Leeuwenhoek **15**, 65, 1949. – 4. J. R. Salversen and D. Hogan, Anal. Chem. **20**, 909, 1948. – 5. F. P. Treadwell, Lehrbuch der analytischen Chemie II, 317, 1949. – 6. H. Zahnd, R. K. Maurmayer and E. M. Livingston, Chemist Analyst **39**, 1, 1950. – 7. C. E. ZoBell, J. Bact. **46**, 39, 1943.

(Hopkins Marine Station, Pacific Grove, California, U.S.A.).

THE EFFECT OF CARBON DIOXIDE ON REDUCTION OF METHYLENE BLUE BY MICRO-ORGANISMS

by

H. GEST [1]) and **J. L. STOKES** [2])

(Received September 15, 1951).

HES (1, 2) has reported that CO_2 accelerated the reduction of methylene blue by *Escherichia coli*, *Serratia marcescens*, *Aspergillus niger* and other microorganisms in the presence of certain substrates. This implies that CO_2 is involved in the dehydrogenation mechanisms of the microbial cell. It seemed possible that CO_2 might function in dehydrogenations by stimulating formation of the hydrogen transporting dicarboxylic acids, oxalacetic, fumaric, malic, etc., through a $C_1 + C_3$ synthesis. Accordingly an attempt was made to substitute dicarboxylic acids for CO_2 in experiments on methylene blue reduction with *E. coli* and other bacteria. In contrast to HES, however, we could not find any effect of CO_2 on methylene blue reduction in the presence of readily utilizable compounds. A CO_2 effect was obtained but, as is shown below, this appears to be associated primarily with the endogenous metabolism of the cell.

EXPERIMENTS.

The experimental procedure of HES was as follows: Thoroughly washed and aerated cells, suspended in phosphate buffer, were placed in Thunberg-Keilin tubes with methylene blue and unspecified hydrogen donors. Where removal of CO_2 was desired, solid

---

[1]) Present address, Department of Microbiology, Western Reserve University School of Medicine, Cleveland, Ohio.

[2]) Present address, Department of Bacteriology, Indiana University, Bloomington, Indiana.

KOH was placed in the side bulb. The tubes were evacuated and shaken at 0° C. for several hours. The low temperature prevented dehydrogenase action during the period of CO_2 absorption by the alkali. Methylene blue reduction was then followed at 37° C. With suspensions of *Escherichia coli*, *Serratia marcescens* (*B. prodigiosum*) and other organisms, Hes found that removal of CO_2 in this manner prolonged the reduction time for as much as 24 hours.

In a number of our experiments in which the Hes technique was employed the same rapid rate of reduction of methylene blue was obtained in the presence or absence of KOH, with suspensions of *E. coli* acting on glucose, malate or lactate. Thus in contrast to Hes' results, CO_2 had no apparent effect on the time of reduction of methylene blue.

Additional experiments were made with a modified procedure employing Warburg vessels in place of Thunberg tubes. Two-tenths ml of 0.0053 M (1 : 500 dilution) methylene blue was placed in one side arm and 0.1 ml of 0.1 M substrate in the other side arm. Two ml of cell suspension containing 15 to 30 cmm of cells in M/15 phosphate buffer at pH 6.2 or 7.0 were placed in the main compartment. For each substrate tested, a pair of vessels was used only one of which contained 0.2 ml of 25% KOH in the center well for absorption of CO_2. Vessels containing KOH were flushed with N_2 for 10 to 15 minutes whereas the other vessels received 5% CO_2 in N_2. The bath temperature was 30° C. Before tipping substrate and methylene blue, the vessels were equilibrated for 30 to 45 minutes to permit further absorption of residual CO_2 by the alkali. Decolorization of methylene blue was followed visually and the time required for complete reduction was noted. Observations were facilitated by the use of a Warburg bath equipped with a glass window beneath the vessels.

Experiments were made with *E. coli* cells grown in yeast extract, 2 per cent glucose medium buffered with 2 per cent of M/1 K_2HPO_4. After approximately 18 hours of growth at 35° C., the bacteria were centrifuged and washed once or twice with distilled water. The washed cells were then suspended in M/15 phosphate buffer of pH 7.

Typical results showing the effect of CO_2 on methylene blue reduction are given in Table 1. Elimination of CO_2 did not delay the reduction of methylene blue by *E. coli* in the presence of glucose, sucrose, malate, citrate, pyruvate, succinate, or lactate. All of

TABLE 1.

Effect of CO_2 on methylene blue reduction by *E. coli*.

Substrate	Reduction time (minutes)		
	With CO_2	Without CO_2	$\triangle t$
None	100	165	+65
None	110	165	+55
Glucose	10	10	0
Sucrose	40	40	0
Lactate	18	18	0
Pyruvate	30	30	0
Succinate	70	70	0
Citrate	73	73	0
Malate	95	90	—5
Fumarate	100	100	0
Acetate	105	>290	>+185
Butyrate	115	214	+ 99
Glycine	120	>165	>+ 45

these compounds, with the possible exception of malate, gave reduction times that were less than those shown by the endogenous controls. Prolongation of the reduction time by CO_2 removal was, however, clearly evident with the endogenous controls and also with acetate, butyrate and glycine. With the latter compounds, the reduction times in the presence of CO_2 were almost identical with those of the endogenous controls. This suggests that hydrogen was not transferred from acetate, butyrate and glycine to the acceptor dye and accordingly, that the reduction of methylene blue was accomplished at the expense of endogenous hydrogen donors. It should be noted that in the absence of CO_2, the reduction times with acetate and butyrate were significantly longer than those shown by the endogenous controls and the CO_2 effect ($\triangle t$) was consequently more pronounced in these instances. The reasons for the apparent inhibition of methylene blue reduction by acetate and butyrate in the absence of CO_2 are not known; one possible explanation is that these acids or their metabolic derivatives may react with, and thereby deplete the level of, carriers required for hydrogen transport from endogenous reserves to methylene blue.

It has been found that the effect of CO_2 on methylene blue reduction by endogenous constituents of the cells can only be observed if the cells are first depleted of excess reserve material and possibly hydrogen carriers either by aeration or dilution. The effect of aeration on the appearance of the CO_2 phenomenon is shown in Table 2.

TABLE 2.
Influence of previous aeration of an *E. coli* suspension on the CO_2 effect.

Hours of aeration	Reduction time (minutes)		
	With CO_2	Without CO_2	$\triangle$ t
0	153	153	0
2	153	153	0
5	203	348	+145
9	290	400	+110

The data were obtained with a suspension of *E. coli* which was aerated with a slow stream of air. Aliquots of the aerated suspension were removed at intervals for testing in the usual manner, in the absence of substrate. The retarding effect of the removal of CO_2 on reduction of methylene blue by the endogenous hydrogen donors was not evident with unaerated cells or those aerated for only two hours. The effect appeared strikingly with cells aerated for five or nine hours; in these cases, the reduction times were increased 145 minutes and 110 minutes respectively by removal of CO_2. Cells aerated for 21 hours failed to completely reduce methylene blue within 10 hours. However the degree of reduction at the end of the experiment seemed to be about the same with and without CO_2. Thus the CO_2 effect was not observed with cells which were insufficiently aerated or those which were aerated for too long a period of time.

TABLE 3.
Influence of dilution of an *E. coli* suspension on the CO_2 effect.

Dilution	Reduction time (minutes)		
	With CO_2	Without CO_2	$\triangle$ t
Undiluted [1]	38	35	— 3
1 : 2	150	355	+205
1 : 4	>1200	>1200	—

---

[1] 15 cmm of cells per ml.

As shown in Table 3, results similar to those obtained by aeration can be obtained by appropriate dilution of the cell suspension. It is evident that the undiluted suspension of *E. coli* did not show the CO_2 effect whereas a 1 : 2 dilution gave rise to the CO_2 phenomenon. With further dilution of the suspension, methylene blue reduction was not observed either in the presence or absence of CO_2 even after 1200 minutes. Comparable results were obtained with the photosynthetic bacterium, *Rhodospirillum rubrum* in that appropriate dilution of a suspension of the organism resulted in the appearance of the CO_2 effect on the reduction of methylene blue by endogenous donors, whereas with the more concentrated suspension no effect was observed.

The influence of dilution as well as aeration may be related, at least in part, to reduction of endogenously produced CO_2 to a critical level. Although KOH was present, it is still possible that the CO_2 was not always completely removed due to the slowness of diffusion of CO_2 from the suspension into the KOH in the center well. Thus it is possible that with a moderately high rate of CO_2 production from endogenous reserves, the CO_2 concentration in the cell suspension remains above the critical level despite the presence of KOH. A second contributing factor may be loss of coenzymes due to dilution or aeration. A combined effect of this nature has been demonstrated by GREENSTEIN and CHALKLEY (3) in connection with dehydrogenase activity in dialyzed mouse liver extracts. Just as in the experiments of HES and in those reported here, an accelerating effect of CO_2 on methylene blue reduction was observed. Addition of both CO_2 and diphosphopyridine nucleotide led to a more pronounced decrease of reduction time than did CO_2 alone.

The effect of 100 micrograms of coenzyme I [1]) on the endogenous reduction of methylene blue by *E. coli* was therefore tested in one experiment. The following reduction times were obtained.

CO_2 absent	
1. No coenzyme	200 minutes
2. 100 μg coenzyme	170 minutes
CO_2 present	
1. No coenzyme	134 minutes
2. 100 μg coenzyme	133 minutes

---

[1]) Obtained through the courtesy of Dr PAUL STUMPF of the University of California. The material, from baker's yeast, was approximately 40% pure and contained a trace of flavin according to Dr STUMPF.

The addition of coenzyme I increased the rate of methylene blue reduction in the absence of CO_2. It did not completely replace CO_2 nor did it decrease the time for methylene blue reduction when CO_2 was present.

In an additional experiment designed to determine the effect of CO_2 on methylene blue reduction by exogenous hydrogen donors, *Acetobacter suboxydans* was used since this organism oxidizes various substrates such as glycerol and glucose incompletely and presumably without the formation of CO_2. Although methylene blue reduction with glycerol and glucose was fairly rapid, no effect of CO_2 on the reduction was discernible. Also, CO_2 had no influence on the very slow endogenous reduction by the cells.

Discussion.

Our results clearly indicate that CO_2 increases the rate of methylene blue reduction or conversely, a much longer time is required for reduction if CO_2 is eliminated from the system. However, this effect of CO_2 has as yet been demonstrated clearly only on the endogenous metabolism. In contrast to the present results, Hes' original reports implied that the CO_2 effect is observable with exogenous hydrogen donors. Our observations can now be rationalized with those of Hes' with the aid of some new information kindly supplied by Hes in a private communication. Hes states that he did not include any endogenous controls in his experiments, i.e., cell suspensions without added substrate. Consequently, his failure to see the effect of CO_2 upon endogenous methylene blue reduction is understandable. Furthermore, Hes used, at least in most of his experiments, formate and acetate as substrates. Although we have no information on formate, our data with acetate indicate that this compound is not dehydrogenated by *E. coli* with methylene blue as hydrogen acceptor. Moreover, as will be shown in the following paper (5) methylene blue is a powerful inhibitor of the oxidation of acetate by O_2. It seems highly probable, therefore, that although Hes believed he was observing the effect of CO_2 on methylene blue reduction by acetate what he actually observed was the effect of CO_2 upon methylene blue reduction by endogenous cell materials.

The need of CO_2 for methylene blue reduction can be readily understood on the basis that CO_2 is required for the formation of more complex carbon compounds such as dicarboxylic acids which are necessary for the transport of hydrogen from endogenous donors to methylene blue. Indeed, there is some indication in our experiments that malate and fumarate can replace the CO_2 function since although the reduction periods with these compounds were essentially the same as that of the endogenous controls in the presence of CO_2 ,the removal of CO_2 failed to increase the reduction times (see table 1).

It is quite possible that there are some exogenous substrates whose rate of dehydrogenation by *E. coli* with methylene blue as acceptor is materially influenced by CO_2. However none of the many compounds we have tested show this behavior. Perhaps some of the readily dehydrogenated compounds would show the CO_2 effect if CO_2 could be continuously and completely removed during the experiment. VAN NIEL (4) has reported the dependence of the oxidation of acetate by *Rhodospirillum rubrum* on CO_2 and has shown that with O_2 as acceptor, this organism oxidizes acetate only when CO_2 is supplied. The CO_2 could be replaced by trace amounts of succinic, fumaric and related acids.

Our results indicate that, in general, readily utilizable compounds provide through their decomposition CO_2 and CO_2-dependent hydrogen transfer systems at a rate sufficiently high so as to make it virtually impossible to deplete the cells to the critical level where the CO_2 effect can come into play. In the absence however of such substrates and therefore of a continuous supply of CO_2 and CO_2-dependent hydrogen transport systems, the available free and bound CO_2 can be reduced sufficiently so that the CO_2 effect is demonstrable. This state is difficult to attain for as both HES and we have shown the cell suspensions must first be depleted of endogenous reserves and of CO_2 by incubation and aeration or dilution.

A practical consequence of our experiments may be emphasized. Reduction rates in dehydrogenase-methylene blue experiments are calculated as time differences in reductions by cell suspensions with and without substrates. It is therefore important that the time for reduction of methylene blue by the endogenous control should not be increased significantly due to lack of CO_2. This may occur if dilute cell suspensions and unduly long evacuation periods are employed. The simplest way of avoiding the complicating effect of CO_2 is to use suspensions sufficiently dense so that there is no CO_2 effect in the absence of substrate.

Summary and Conclusions.

The experiments of Hes which indicate that CO_2 is essential for the reduction of methylene blue by microorganisms presumably because it is required for the functioning of the cellular hydrogen transport mechanisms, have been repeated and results have been obtained that seemingly differ from those of Hes. We could not demonstrate any effect of CO_2 on the rate of methylene blue reduction by various bacteria in the presence of utilizable compounds such as glucose, sucrose, lactate and pyruvate. Using *E. coli* and other bacteria we found, however, that the time required for the reduction of methylene blue was greatly increased by CO_2 removal when no substrates were added. This effect of CO_2 on the endogenous metabolism was observed only when the cells were depleted of endogenous reserves and of CO_2 by aeration or dilution.

We are indebted to Dr C. B. van Niel for his advice and help.

References.

1. J. W. Hes, Nature **141,** 647, 1938. – 2. J. W. Hes, Ann. Fermentations **4,** 547, 1938. – 3. J. P. Greenstein and H. W. Chalkley, J. Biol. Chem. **160,** 371, 1945. – 4. C. B. van Niel, Photosynthesis in Plants, edited by J. Franck and W. E. Loomis, 468, 1949. – 5. J. L. Stokes, Antonie van Leeuwenhoek **18,** 63, 1952.

(Hopkins Marine Station, Pacific Grove, California, U.S.A.).

INHIBITION OF MICROBIAL OXIDATION, ASSIMILATION AND ADAPTIVE ENZYME FORMATION BY METHYLENE BLUE

by

J. L. STOKES 1)

(Received September 15, 1951).

It has been thoroughly established that non-proliferating cell suspensions of bacteria, yeasts and other microorganisms generally cause an oxidative decomposition of utilizable substrates such as alcohols, organic acids, carbohydrates, etc. which does not yield the theoretically maximum amount of CO_2 and H_2O. The oxidation is incomplete, and part of the substrate — which may vary from 25 % to 85 % — is converted into cellular material, the latter having approximately the empirical composition of a carbohydrate, $(CH_2O)_n$. This process of oxidative assimilation has been observed with all microorganisms so far examined and also with all substrates tested with the sole exception of formate which is completely oxidized (*cf.* Clifton (1), for an excellent review).

Shortly after most of the principal features of oxidative assimilation were described by Barker (2), Clifton (3) showed that the assimilatory process in *Escherichia coli*, *Spirillum serpens* and *Pseudomonas calcoacetica* could be prevented by sodium azide (NaN_3), α-2,4-dinitrophenol (DNP) and monoiodoacetic acid. Addition of M/70,000 to M/600 of these poisons to the cell suspensions resulted in the complete or almost complete oxidation of acetate and butyrate to CO_2 and H_2O apparently without the temporary accumulation of assimilation products. Subsequently, the inhibition of oxidative and fermentative assimilations by these cellular poisons, especially NaN_3 and DNP, was demonstrated for other microorganisms and other substrates by Clifton and Logan

---

1) Present address: Department of Bacteriology, Indiana University, Bloomington, Indiana.

(4), Winzler (5), Doudoroff (6), Burris and Wilson (7), Pickett and Clifton (8, 9), van Niel and Anderson (27) and Reiner and Spiegelman (10) so that now this phenomenon is as well established as that of assimilation itself. In line with this effect on primary assimilation are the observations which show that NaN_3 and DNP also prevent the formation of adaptive enzymes and in general tend to block synthetic processes [Monod (11), Spiegelman (12)]. Although the precise mechanisms by which these poisons exert their effects still remain to be elucidated, the investigations of Hotchkiss (13), Loomis and Lipmann (14,15) and Spiegelman, Kamen and Sussman (16) have shown that azide and dinitrophenol apparently inhibit esterification of inorganic phosphate without interfering with glycolysis or respiration. Thus they seem to dissociate or uncouple hydrogen transfer reactions from the generation of high energy phosphate bonds. Since such bonds are generally considered to be the primary source of energy for biological syntheses (Lipmann (17, 18)), it becomes understandable why NaN_3 and DNP prevent assimilation and other synthetic processes.

In the present report it is shown that also methylene blue is a powerful inhibitor of oxidative assimilation and adaptive enzyme formation. In addition, these studies reveal that such methylene blue inhibitions exhibit a number of properties that are quite different from those of azide and dinitrophenol.

MATERIALS AND METHODS.

A variety of microorganisms, including bacteria, yeasts and a colorless alga have been used in the form of cell suspensions for these experiments. The methods of obtaining the suspensions follows.

Suspensions of *Escherichia coli* were prepared by first growing the cells either on yeast extract agar or in shallow layers of yeast extract broth for 12 to 18 hours at 30°C. The cells were then harvested, washed once with water and suspended in sufficient M/15 phosphate buffer at pH 7.0 to give a concentration of 1.5 mg of cells, dry weight, per ml. The suspension was aerated gently for 24 hours at room temperature to reduce endogenous reserves, centrifuged and resuspended in an equal amount of fresh buffer. Unused portions were stored in the refrigerator and discarded if not used within a week.

Pseudomonas calcoacetica, for some unknown reason, grew poorly in shallow layers of yeast extract broth. On agar plates of the same medium growth was more abundant but viscous. This latter difficulty was circumvented by drying the plates prior to inoculation. The cells were harvested after 1 day at 30°C., washed once with water and used in the manometric experiments without preliminary aeration.

Pseudomonas putida was grown on yeast extract agar medium for 1 day, harvested, washed twice with water and resuspended in sufficient M/15 phosphate buffer at pH 7.0 to give a reading of 340 in the Klett photoelectric colorimeter at a wave length of 660 mμ.

Prototheca zopfii, *Saccharomyces cerevisiae* and *Torulopsis monosa* were grown on yeast extract, 2 per cent glucose agar plates at 30°C. for 1 or 2 days, harvested, washed twice with water and suspended in sufficient M/10 or M/15 phosphate buffer at the desired pH to provide 10 μl of cells per ml as measured in Hopkins tubes.

The Barcroft-Warburg apparatus was used for the manometric experiments. Each vessel received 2 ml of cell suspension. 5 or 10 μM of substrate dissolved in 0.1 or 0.2 ml of water were placed in the side arm as was also the methylene blue solution. When oxygen consumption alone was to be determined, 0.2 ml of 10 per cent KOH was placed in the center well to absorb CO_2. Organic acids as substrates were used as the Na-salts. The gas phase was air, N_2, or $N_2 + 5$ per cent CO_2 as necessary. The bath temperature was 30°C. Methylene blue reduction tests were carried out in the Warburg vessels in an atmosphere of N_2. The Q_{O_2} values represent the microliters of O_2 consumed per mg of cells (dry wt.) per hour. Additional details of procedure will be given at appropriate places in the text.

Experiments.

The experiments reported in this paper originated from the unexpected observation that suspensions of *E. coli* which rapidly oxidized acetate in the presence of oxygen failed to reduce methylene blue (MB) when supplied with acetate under N_2. Generally, with strictly aerobic or facultatively aerobic microorganisms both acceptors are equally effective whenever the substrate can be metabolized. A more detailed study of this phenomenon led to the discovery that certain oxidative and synthetic reactions are inhibited by MB.

1. Inhibition of oxidation by MB.

The data in table 1 illustrates the anomalous behavior with respect to the oxidation of acetate. It is evident that *E. coli* rapidly consumes O_2 and also quickly decolorizes MB when glucose, pyruvate or lactate is the substrate. With acetate, however, although the rate of O_2 uptake is essentially equal to that observed with glucose and pyruvate, and considerably greater than that with lactate, yet there is no evidence of MB reduction which cannot be accounted for as due to dehydrogenations of endogenous cell material.

TABLE 1.
Comparison of oxygen consumption and methylene blue reduction with various substrates by *E. coli*.

Substrate	Q_{O_2}	Minutes required for complete reduction of 1 μM MB
Acetate	61	88
Glucose	69	5
Pyruvate	67	25
Lactate	40	15
Endogenous	4	88

There are two possible explanations for the lack of MB reduction with acetate. Either the dye is not reduced because the oxidation of acetate obligatorily requires oxygen or else MB in the amount used (1 micromole) is toxic for dehydrogenation of acetate although apparently not for that of glucose, pyruvate and lactate. The validity of the first alternative is dependent upon the elimination or establishment of toxicity. If MB were toxic one might expect that lowering the concentration would result in a smaller degree of inhibition. But decreasing the amount of MB to as little as 0.2 μM per vessel failed to produce more rapid reduction with acetate than was obtainable without acetate. This could be taken to mean that toxic effects are not involved. However, with such small amounts of MB, reduction is complete within 5 to 10 minutes which is too short an interval for accurate determination of rates. By a different experimental approach, strong evidence was obtained that MB is toxic rather than inert. If 1 μM of MB is added together with acetate to *E. coli* suspensions exposed in air, the consumption of oxygen, which occurs rapidly in the absence of MB, is now completely inhibited. Amounts of MB smaller than 1 μM produce, in almost direct proportion, less inhibition of oxygen uptake. It can be seen in figure 1 that with a cell suspension having a Q_{O_2} of 61 for acetate, this value is reduced 31 per cent by 0.25 μM, 74 per cent by 0.5 μM, and 100 per cent, to the level of the endogenous rate, by 1 μM MB.

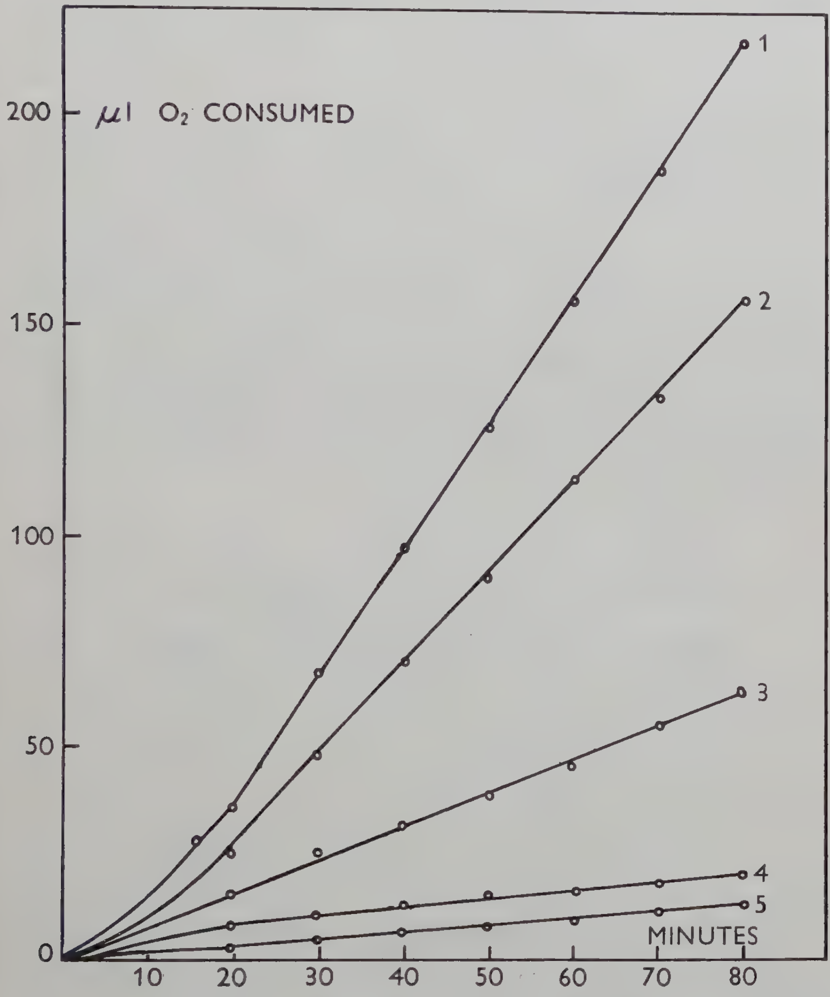


Figure 1. Inhibition of acetate oxidation by *E. coli* with methylene blue (MB). 1 = No MB, Q_{O_2} 61; 2 = 0.25 μM MB, Q_{O_2}42; 3 = 0.5 μM MB, Q_{O_2}16; 4 = 1 μM MB, Q_{O_2}4; 5 = endogenous, Q_{O_2}5.

In striking contrast, 1 μM MB not only did not repress oxidation of glucose, pyruvate and lactate but rather stimulated the rate of oxygen consumption by 10 to 20 per cent. Inhibition of acetate oxidation by MB was consistently obtained in many experiments. It is due to MB itself rather than to impurities since identical results were obtained with a sample of MB which had been recrystallized six times from ethanol.

The efficacy of MB as inhibitor of acetate oxidation is inversely related to the rapidity with which a particular suspension can oxidize acetate; oxidation by a suspension having a Q_{O_2} of 45 was completely repressed by as little as 0.5 μM MB whereas 1 μM MB reduced the rate of oxidation by one with a Q_{O_2} of 85 by only 50 %. These quantitative relations emphasize the specific nature of the inhibitory effect. At a concentration of MB sufficient to give about 50 per cent inhibition, the inhibitory effect is independent of the concentration of acetate in the range of 5 to 40 micromoles. Likewise, MB inhibition is largely independent of pH in the range of 5.3 to 8.1 as shown in table 2. There is a trend towards less inhibition with increase in pH but this is not very marked. Keilin (19) has shown that pH has a decisive effect upon the inhibition of glucose oxidation by yeast with NaN_3; respiration was completely suppressed by azide at pH 5.6 but not at all affected at pH 7. Also, Field, Martin and Field (20, 21) have shown that the stimulation and inhibition of yeast respiration by DNP is a function of pH and is greatest at acid reactions which favor the existence of the undissociated DNP. MB differs therefore from NaN_3 and DNP in

TABLE 2.

Effect of pH on methylene blue inhibition of acetate oxidation by *E. coli*.

pH	Acetate Q_{O_2}	Acetate + 0.5 μM MB Q_{O_2}	Inhibition Per cent
5.3	31	9	71
6.5	48	16	67
7.0	47	15	68
7.5	51	25	51
8.1	54	23	57

that its inhibitory effect on respiration is relatively independent of pH.

Since the presence of MB prevents the oxidation of acetate by *E. coli* when oxygen is used as the major or final hydrogen acceptor, it seems reasonable to assume that also the anaerobic oxidation with MB as acceptor is inhibited by the dye through a similar or even identical mechanism. The interference would seem to involve specifically the enzymatic activation of acetate because, as was previously shown, for the oxidation of substrates other than acetate, *E. coli* can utilize oxygen and MB equally well as hydrogen acceptors.

In marked contrast to the sensitivity of the oxidation of acetate to MB, the oxidation of glucose, glycerol, lactate and pyruvate is very much less influenced by it. Usually 2 μM MB did not affect the rates of oxidation of these compounds at all whereas 1 μM sometimes even increased the rates. The results with higher concentrations of MB are shown in table 3. Ten micromoles of MB failed to completely suppress the oxidation of glycerol and inhibited the rate of oxidation of pyruvate by only 23 per cent whereas 25 μM of MB actually increased the rate of oxidation of glucose.

TABLE 3.

Effect of methylene blue concentration on rate of oxidation of glucose, glycerol and pyruvate by *E. coli*.

Micromoles of MB	Glucose Q_{O_2}	Glycerol Q_{O_2}	Pyruvate Q_{O_2}
0	113	96–130	78
4		96	58
10	134	28	60
25	127	12	

Now one might expect that the above-mentioned substrates are oxidized by the commonly accepted pathways, i.e. terminally through acetate and the tricarboxylic acid cycle. The occurrence of such a mechanism should bring into play the inhibitory effect of MB discussed above. That MB in the reduction tests did not appear toxic is of course understandable because the substrates must undergo dehydrogenations for which MB can be acceptor and which are not inhibited by the dye, prior to acetate formation. With pyruvate, the phosphoclastic reaction would give rise to acetate and formate [Utter, Lipmann and Werkman (22); Stokes (23)] and the hydrogen of the latter would reduce MB through activation by formic dehydrogenase (Gale (24)) which is known to be present in most coli cells and also in the particular strain used in these experiments. In the dissimilation of glucose, hydrogen in addition to that from pyruvate would be made available during the oxidation of phosphoglyceraldehyde to phosphoglyceric acid; and from lactate during its oxidation to pyruvate. Similar considerations apply to glycerol. Since 10 μM of each compound were used, 10 to 40 μM of hydrogen would be potentially available for the reduction of MB. These quantities are greatly in excess of the 1 μM of hydrogen which is necessary for the total reduction of the 1 μM of MB used.

But the implication of the postulated degradation of glucose, glycerol, lactate and pyruvate via acetate ought to be that oxidation of these substrates in the presence of MB should result in the accumulation of acetate. This was first tested for glucose oxidation. To provide sufficient material for chemical analyses, 100 ml of coli suspension prepared in the usual fashion and 500 μM of glucose were placed into each of two 1-liter erlenmeyer flasks, one of which also received 100 μM of MB. Previous "titration" of the suspension had shown that 1.5 or 2 μM of MB per 2 ml of suspension completely suppressed the oxidation of added acetate. The open flasks were incubated at 30° C. on a shaking machine. Simultaneously, a manometric experiment was set up at 30° C. with air as the gas phase, on 1/50th the scale of the main experiment, to provide information on the rates of oxygen consumption and the time of completion of the oxidations. The glucose and MB were added to the large flasks immediately after tipping these ingredients into the Warburg vessels.

The rates of oxygen consumption showed a sharp decrease after about 4 hours (figure 2) and at this time the large flasks were removed from the incubator. The contents were brought to pH 2 with H_2SO_4, the cells removed by centrifugation, and aliquots of the supernatants were steam distilled to determine whether volatile acids were present. No volatile acid was found in the supernatant of the suspension which had not received MB. But the one with the dye contained a total of 560 micromoles of acid, equivalent to 1.12 moles per mole of glucose oxidized. The acid was unequivocally identified as acetic acid by Duclaux distillation. The accumulation of acetic acid therefore fulfills expectations. The amount of acetic acid is, however, less than that which could have been expected, *viz.*, 2 mols per mole of glucose. In two other experiments of the same sort, 0.86 and 1.43 moles of acetic acid were found to have accumulated per mole of glucose decomposed. The variations observed are not easily accounted for, but the low yield of acetic acid would seem to imply that either the oxidation of glucose need not completely pass through acetic acid as an intermediate product, or that in actively metabolizing cell suspensions the effect of MB

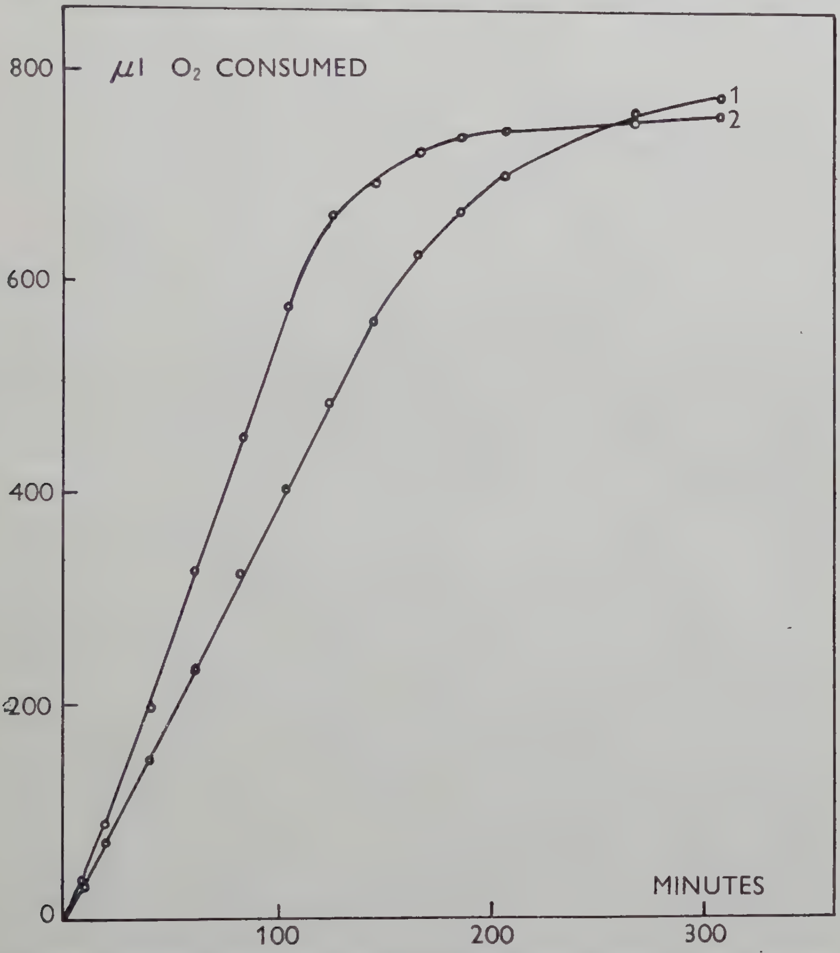


Figure 2. Effect of methylene blue (MB) upon the oxidation of 10 µM glucose by *E. coli*. 1 = glucose; 2 = glucose plus 2 µM MB.

on acetate oxidation is somehow not exerted to the full extent.

This is also indicated by similar experiments on the oxidation of glycerol, lactate and pyruvate by *E. coli* in the presence of MB. In such systems it has so far not been possible to detect the accumulation of acetic acid. Yet it seems reasonable, on the basis of our present knowledge of such oxidations, to anticipate that they would proceed via the intermediate formation of acetic acid.

Further analysis of the foregoing data, later supported by additional experiments, disclosed a new aspect of the biological activity of MB, *i.e.* its ability, at concentrations which need have no effect upon oxidation rates, to inhibit synthetic processes.

2. Inhibition of oxidative assimilation.

Examination of the oxygen consumption curves for the manometric experiment (figure 2) would lead one to believe, in the absence of chemical data, that the only effect of MB on glucose oxidation was a slight increase in the rate of oxidation. Yet, as we have seen, the influence of the dye is much more profound since it results in the accumulation of a readily detectable product of incomplete glucose oxidation. This very fact raises the question why the amounts of oxygen consumed for the oxidation of glucose in the presence and in the absence of MB are so very similar.

The complete oxidation of 10 μM glucose, according to the equation $C_6H_{12}O_6 + 6O_2 \rightarrow 6\ CO_2 + 6\ H_2O$ requires $6 \times 224\ \mu l = 1344\ \mu l\ O_2$. But the rate of O_2 consumption decreased to a level approaching that of endogenous oxidation when only an amount of between 700 and 780 μl (no MB) or 740—760 μl (in presence of MB) had been used. The former figures represent only about 55 per cent of the oxygen required for the complete oxidation of the glucose. But this was to be expected since CLIFTON and LOGAN (4) have already shown that under these conditions *E. coli* assimilates about 50 per cent of the glucose and that the oxidation can be represented by the equation

$$C_6H_{12}O_6 + 3\ O_2 \rightarrow 3(CH_2O) + 3\ CO_2 + 3\ H_2O$$

Likewise, the figures obtained with MB also represent about 55 per cent of the oxygen required for the complete oxidation of the glucose. In this case, however, the remaining 45 per cent or about 600 μl O_2 cannot be accounted for by assimilation products, (CH_2O), since 11.2 μM of acetic acid had accumulated. The complete oxidation of this amount of acetic acid according to the equation $CH_3COOH + 2\ O_2 \rightarrow 2\ CO_2 + 2\ H_2O$ requires $11.2 \times 2 \times 22.4\ \mu l =$ 502 μl O_2. Subtraction of this amount from the 600 μl noted above leaves only 98 μl O_2. This means that only about 5 per cent of the glucose could have been assimilated compared to the 45 per cent in the oxidation without MB.

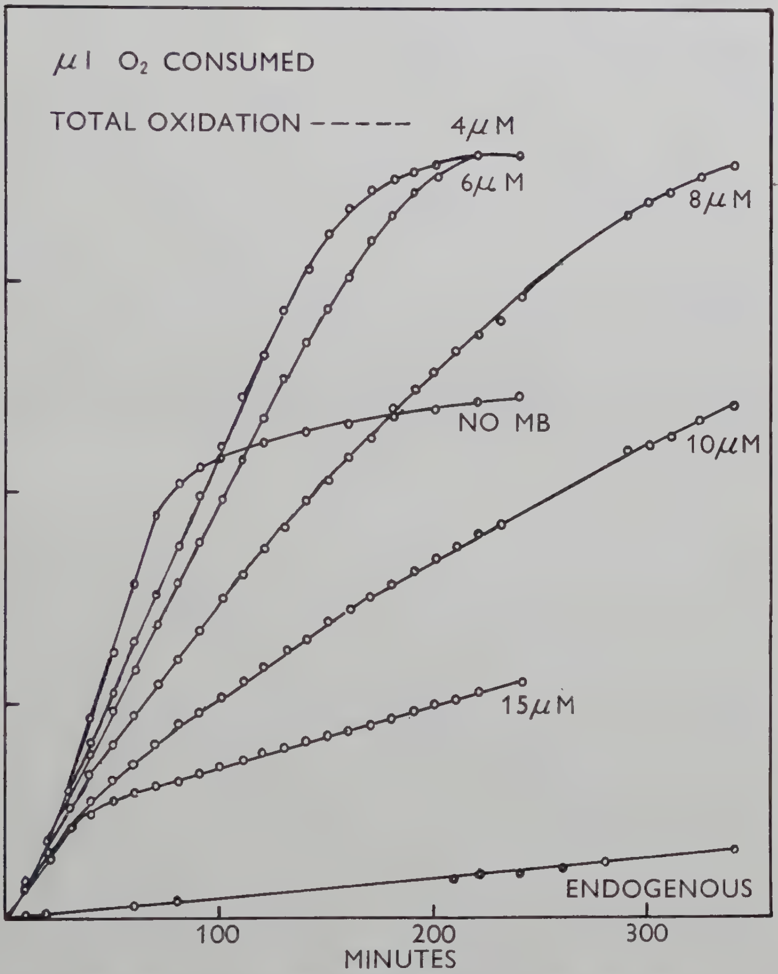


Figure 3. Inhibition by methylene blue (MB) of the assimilation and oxidation of glycerol (9.6 μM) by *E. coli.*

The conclusion is inescapable that MB suppressed assimilation by *E. coli* almost completely during the oxidation of glucose. This unexpected property of MB could readily be demonstrated with other substrates.

Thus the complete oxidation of 9.6 μM of glycerol according to the equation $C_3H_8O_3 + 3.5\ O_2 \rightarrow 3\ CO_2 + 4\ H_2O$ requires $9.6 \times 3.5 \times 22.4\ \mu l = 753\ \mu l\ O_2$. The observed value in the absence of MB (figure 3) at the time that the rate of oxygen consumption had decreased to a level approaching that of endogenous oxidation was 428 $\mu l\ O_2$. This is 57 per cent of the amount required for complete oxidation and is exactly the amount to be expected from the equation $2\ C_3H_8O_3 + 4\ O_2 \rightarrow 3(CH_2O) + 3\ CO_2 + 5\ H_2O$ established by CLIFTON and LOGAN (4) for the oxidation of glycerol by *E. coli*. However with 4, 6 and 8 μM MB the amounts of oxygen consumed were 706, 712 and 712 μl respectively or 94 to 95 per cent of the amount required for complete oxidation of the glycerol.

Figure 3 also shows that the rate of glycerol oxidation decreases progressively as the MB concentration is increased from 4 to 15 μM. This inhibition of oxidation by MB is analogous to that observed in the oxidation of acetate (figure 1). Therefore the dye has a two-

TABLE 4.

Inhibition of oxidative assimilation and rate of oxidation by *E. coli* as a function of methylene blue concentration.

Substrate	Oxygen consumed	Micromoles of methylene blue									
		0	0.01	0.05	0.1	0.2	0.5	1	2	4	10
Pyruvate	Per cent[1]	52			75		92	83		59	46
	Q_{O_2}	78			82		87	74		58	60
Lactate	Per cent	69				106	87	57	24	22	
	Q_{O_2}	27				28	28	28	20	18	
Acetate	Per cent	75	84	104	102		119		12		
	Q_{O_2}	33	33	37	27		20		4		

[1] $$\frac{\mu l\ O_2\ \text{consumed}}{\mu l\ O_2\ \text{required for complete oxidation}} \times 100$$

fold effect. At low concentrations it prevents oxidative assimilation and at higher concentrations it inhibits the rate of oxidation.

These two effects of MB were obtained also in the oxidation of pyruvate, lactate and acetate (table 4). Appropriate concentrations of MB prevented oxidative assimilation with the three substrates. Even with acetate, the oxidation of which is so sensitive to inhibition by MB, assimilation was completely prevented by .05 μM of MB. The value of 119 per cent obtained with 0.5 μM MB indicates that oxidation of endogenous material proceeded concomittantly with that of acetate. Similarly high values have been obtained by PICKETT and CLIFTON (9) with *Staphylococcus albus* and *Proteus vulgaris*, and by WINZLER (5) with *Saccharomyces cerevisiae* when assimilation was suppressed by DNP. The endogenous O_2 consumption has not been subtracted from the values given in the table because of the well known difficulty of determining whether endogenous oxidation is proceeding simultaneously with that of the substrate. The endogenous rate, however, has always been low because of the practice of depleting reserves by extended aeration of the cells prior to their use. MB tended to increase the rate of endogenous oxidation by as much as 50 to 100 per cent immediately after being tipped into the suspension but within 20 to 30 minutes, the rate would decrease to the level or even below that of the suspension without dye.

There is, on the whole, no correlation between the rate and the extent of oxidation. MB may prevent assimilation and at the same time fail to have any influence on the rate or else increase or decrease it. However, when the rate is greatly depressed by the dye, the total oxygen consumption also is drastically reduced. This general picture is strikingly similar to that obtained with DNP and azide in the case of *E. coli* and many other microorganisms (*cf.* CLIFTON (1)).

Lacking simultaneous measurements, it is impossible to make exact comparisons of the potencies of MB, DNP and NaN_3 to prevent assimilation. For *E. coli*, CLIFTON and LOGAN found that assimilation of glycerol and pyruvate was almost completely eliminated by M/2000 DNP or M/200 NaN_3. MB inhibited assimilation of glycerol appreciably at M/4000 and pyruvate partly at M/20,000 and almost completely at M/4000. The oxidative assimilation of acetate by *Ps. calcoacetica* (CLIFTON (3)) was inhibited completely by M/8000 DNP or M/600 NaN_3 whereas in our experiments, MB at a concentration of M/40,000 completely prevented assimilation of acetate by *E. coli.* With respect to glucose, Pickett and Clifton (9) obtained partial or complete repression of assimilation by a wide variety of bacteria usually with M/2000 DNP although as little as M/40,000 was effective with *Proteus vulgaris*; with NaN_3, M/400 to M/100 concentrations were required. In our experiments MB was partially effective at M/40,000 and maximally so at M/4000. Comparisons are rendered more difficult by the fact that the activity of DNP and NaN_3 is tremendously influenced by pH whereas this is not the case with MB. In general, it would appear that under the customary conditions of assimilation experiments with bacteria i.e. in phosphate buffer at pH 7, MB is much more potent than NaN_3 and probably somewhat more powerful than DNP as an inhibitor of oxidative assimilation.

In addition to preventing assimilation, MB can also "recover" assimilated material. That is to say, the addition of the dye to cell suspensions after oxidation and assimilation of substrate is completed, causes an immediate spurt in oxygen consumption which continues until the total amount of O_2 taken up approaches the value for complete oxidation. Results of the delayed addition of MB upon the oxidation of 5 μM each of glycerol and pyruvate by *E. coli* are shown in figure 4. A sharp decrease in rate occurred with both substrates after about 80 minutes which indicated that the oxidations were finished. At this time, 51 per cent of the oxygen required for the complete oxidation of the glycerol and 67 per cent for that of the pyruvate had been consumed. After waiting an additional 20 minutes to be certain that oxidation had ceased, 1 μM of MB was tipped into each vessel. The rate of oxygen uptake immediately increased to a level close to that of the initial rate and continued, although with some decrease, until oxygen consumption with glycerol was 82 per cent of that for complete oxidation and with pyruvate, 89 per cent. The experiment was discontinued while the oxidation rate was still high for glycerol so that the figure of 82 per cent is lower than that which undoubtedly could have been obtained. Also the endogenous oxidation was stimulated by the delayed addition of MB but to a much smaller extent than with the substrates.

Similarly, the delayed addition of DNP has been shown by Burris and Wilson (7) to stimulate the oxidation of material assimilated by *Rhizobium* strains during the oxidation of glucose.

But unlike MB, DNP and also NaN_3 are virtually inactive for *E. coli* [Burris and Wilson (7); Clifton (1)].

MB will suppress oxidative assimilation also in organisms other than *E. coli*. For *Pseudomonas calcoacetica* whose oxidative metabolism has been investigated by Clifton (3), we found that in the oxidation of glycerol, 51 per cent of the oxygen necessary for complete oxidation was consumed and that this figure was increased to 80 and 95 per cent by the addition of 0.5 and 1 μM of MB, respectively. Because the cells rapidly lost their oxidizing capacity on storage, they were not aerated prior to use. Therefore the endogenous oxidation with these cells was greater than with *E. coli* and it was considerably increased by MB. If the endogenous oxygen values are subtracted, the above percentages become 29, 44 and

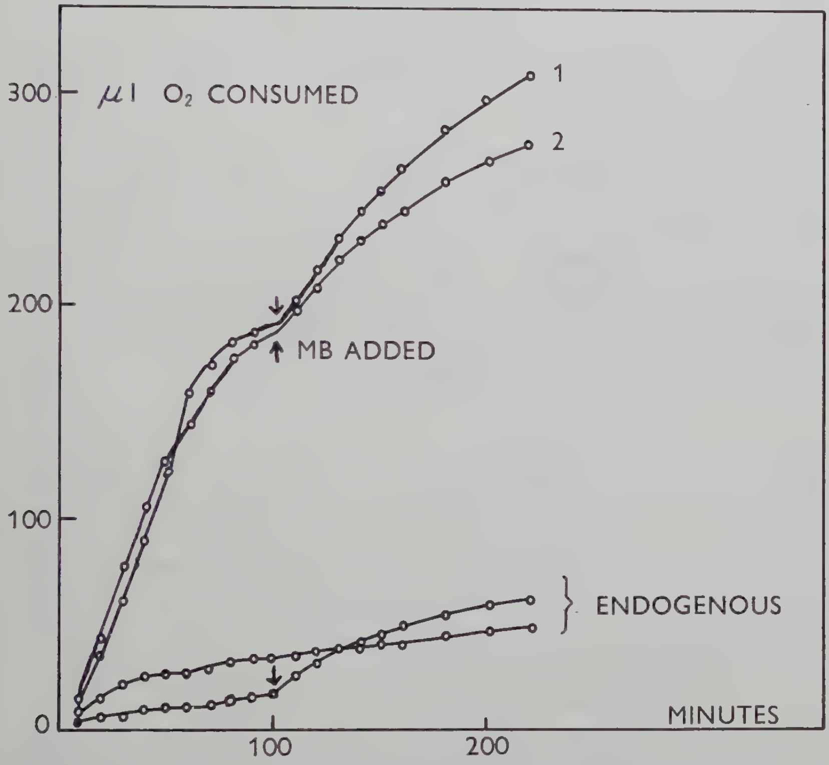


Figure 4. "Recovery" of material assimilated by *E. coli* during the oxidation of 5 μM each of glycerol and pyruvate, by the delayed addition of 1 μM methylene blue. 1 = glycerol; 2 = pyruvate.

59 respectively. Similar results were obtained when acetate was the substrate. Thus the inhibition of oxidative assimilation by MB is clear but the extent is somewhat uncertain.

With *Prototheca zopfii* whose oxidative metabolism has been investigated by BARKER (2) and ANDERSON (25), we obtained, in the oxidation of glycerol, a consumption of oxygen equivalent to 28 per cent of that required for complete oxidation. This figure agrees closely with BARKER's average value of 29 per cent. The addition of 0.1, 0.3, 0.5 and 5.0 μM MB increased the 28 per cent figure to 33, 39, 52 and 66 per cent respectively. Similar results were obtained also with glucose.

However in contrast to the results with the bacteria and the colorless alga, MB was unable to inhibit the assimilation processes of yeasts. Thus in the oxidation of acetate and glucose by *Saccharomyces cerevisiae* at pH 7.0, oxygen consumption was, respectively, two-thirds and one-third of the amounts required for complete oxidation of the substrates, in agreement with the data of WINZLER (5) and of CLIFTON and PICKETT (8) and CLIFTON (26). However, the dye, in concentrations ranging from 0.02 to 5 μM, failed to increase the amounts of oxygen consumed and only depressed considerably the rate of oxidation if more than 1 μM was used. Similar results were obtained with glucose also at pH 4.6 and pH 8.1. Nor could any evidence be obtained with either *S. cerevisiae* or *Torulopsis monosa* that MB inhibits the fermentative assimilation which occurs with yeasts (VAN NIEL and ANDERSON (27)) and which can be prevented by DNP and NaN_3 [WINZLER (28); REINER and SPIEGELMAN (10)].

3. Inhibition of adaptive enzyme formation.

As previously mentioned, DNP and NaN_3 can not only inhibit assimilation but also another synthetic process — the formation of adaptive enzymes. Thus MONOD (11) has shown that DNP inhibits adaptation of *E. coli* to the decomposition of lactose and xylose. Likewise, DNP and also NaN_3 can prevent adaptation of yeasts to the fermentation of galactose [REINER (29); SPIEGELMAN (12)]. It was desirable therefore to determine whether also MB can prevent the formation of adaptive enzymes.

One enzyme system chosen for testing was formic hydrogenlyase which decomposes formic acid into H_2 and CO_2 and which is adaptive in nature (STEPHENSON and STICKLAND (30)). To obtain unadapted cells, *E. coli* was grown under aerobic conditions in yeast extract broth (STOKES (23)). Such unadapted cells will readily form hydrogenlyase as indicated by the production of H_2, if they are incubated with casein hydrolysate, or other complex nitrogenous

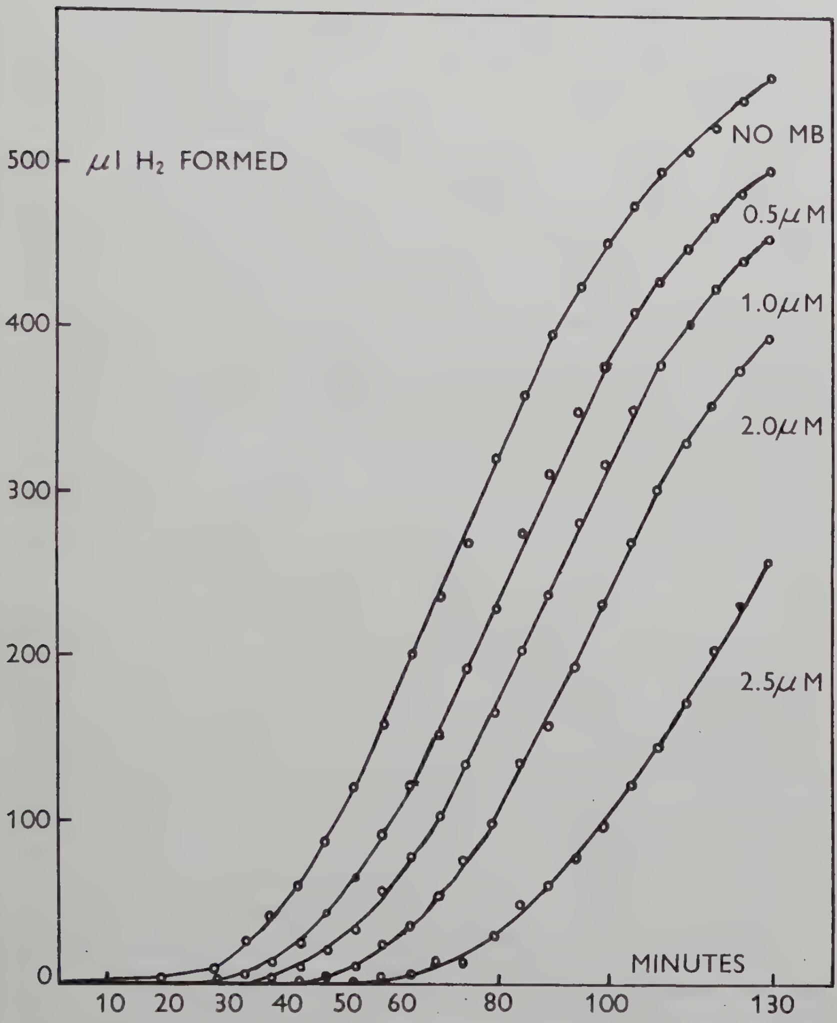


Figure 5. Inhibition of formic hydrogenlyase formation by *E. coli* with methylene blue.

Each vessel received 2 ml of cells suspended in M/15 phosphate buffer, pH 7.0, 10 μM sodium formate, 10 μM glucose, 0.1 ml of a 10 percent solution of Bacto brain heart infusion and the indicated amount of methylene blue. The center well contained KOH and the gas phase was nitrogen.

materials, glucose and formate under anaerobic conditions (PINSKY and STOKES (31)).

MB delayed the synthesis of formic hydrogenlyase by *E. coli* (figure 5); 0.5, 1.2 and 2.5 μM of the dye increased the time necessary for maximum enzyme formation by 11, 19, 30 and 56 minutes, respectively. The possibility that MB inhibits the activity of hydrogenlyase rather than its formation is ruled out by the fact that the same maximum rates were obtained with and without the dye. MB will reduce or completely suppress the activity of the enzyme but only if amounts greater than 3 μM are used. Inhibition of the synthesis of hydrogenlyase by MB is specific since even 5 μM of the dye did not affect the rate or extent of the oxidation or fermentation of glucose under the same conditions.

MB also inhibited adaptation of *Pseudomonas putida* to the oxidation of benzoate (STANIER (32)). This enzyme system was more sensitive to the dye than that of formic hydrogenlyase so that even as little as 0.1 μM delayed maximum enzyme formation by 35 minutes whereas 2 μM delayed adaptation for at least 240 minutes.

Therefore MB, like DNP and NaN_3 can prevent the synthesis of adaptive enzymes.

Summary.

Suspensions of *Escherichia coli* oxidize acetate when oxygen is the hydrogen acceptor but not when methylene blue (MB) is the acceptor. Furthermore, small amounts of MB can completely prevent the aerobic oxidation of acetate. It is concluded, therefore, that MB inhibits the enzymatic activation of acetate.

MB also inhibits the aerobic oxidation of glucose, glycerol, lactate and pyruvate. However at least ten times as much MB are required for inhibition with these substrates as with acetate.

At concentrations of MB which have little or no effect upon the rate of oxidation, the dye suppressses oxidative assimilation by *Escherichia coli*, *Pseudomonas calcoacetica* and the colorless alga, *Prototheca zopfii* but not by the yeasts, *Saccharomyces cerevisiae* and *Torulopsis monosa*.

MB also inhibits adaptive enzyme formation. This has been demonstrated with formic hydrogenlyase of *Escherichia coli* and the benzoate oxidation system of *Pseudomonas putida*.

The biological properties of MB are similar to those of sodium azide (NaN_3) and α-2,4-dinitrophenol (DNP) since, like the latter two compounds, the dye can suppress oxidation, assimilation and adaptive enzyme formation. But the activity of MB differs from that of NaN_3 and DNP in that the inhibition of oxidation by the dye is relatively unaffected by pH; also, MB does not seem to prevent assimilations by yeasts. Quantitatively, MB appears to be much more potent than NaN_3 and at least as active as DNP.

Acknowledgements.

I am greatly indebted to Dr C. B. van Niel for his advice and help during the course of these investigations. I also acknowledge with many thanks the enthusiastic assistance of Dr John Ingraham in some of the experiments.

References.

1. C. E. Clifton, Adv. Enzymology **6,** 269, 1946. – 2. H. A. Barker, J. Cell. and Comp. Physiol. **8,** 231, 1936. – 3. C. E. Clifton, Enzymologia **4,** 246, 1937. – 4. C. E. Clifton and W. A. Logan, J. Bact. **37,** 523, 1939. – 5. R. J. Winzler, J. Cell. and Comp. Physiol. **15,** 343, 1940. – 6. M. Doudoroff, Enzymologia **9,** 59, 1940. – 7. R. H. Burris and P. W. Wilson, J. Cell. and Comp. Physiol. **19,** 361, 1942. – 8. M. J. Pickett and C. E. Clifton, J. Cell. and Comp. Physiol. **21,** 77, 1943. – 9. M. J. Pickett and C. E. Clifton, J. Cell. and Comp. Physiol. **22,** 147, 1943. – 10. J. M. Reiner and S. Spiegelman, J. Cell. and Comp. Physiol. **30,** 347, 1947. – 11. J. Monod, Ann. Inst. Pasteur **70,** 381, 1944. – 12. S. Spiegelman, J. Cell. and Comp. Physiol. **30,** 315, 1947. – 13. R. D. Hotchkiss, Adv. Enzymology **4,** 153, 1944. – 14. W. F. Loomis and F. Lipmann, J. Biol. Chem. **173,** 807, 1948. – 15. W. F. Loomis and F. Lipmann, J. Biol. Chem. **179,** 503, 1949. – 16. S. Spiegelman, M. D. Kamen and M. Sussman, Arch. Biochem. **18,** 409, 1948. – 17. F. Lipmann, Adv. Enzymology **1,** 99, 1941. – 18. F. Lipmann, Adv. Enzymology **6,** 231, 1946. – 19. D. Keilin, Proc. Roy. Soc. (London) Series B, **121,** 165, 1936. – 20. J. Field, A. W. Martin and S. M. Field, J. Cell. and Comp. Physiol. **4,** 405, 1934. – 21. J. Field, A. W. Martin and S. M. Field, Proc. Soc. Exp. Biol. Med. **82,** 1043, 1935. – 22. M. F. Utter, F. Lipmann and C. H. Werkman, J. Biol. Chem. **158,** 521, 1945. – 23. J. L. Stokes, J. Bact. **57,** 147, 1949. – 24. E. F. Gale, Biochem. J. **33,** 1012, 1939. – 25. E. H. Anderson, J. Gen. Physiol. **28,** 297, 1945. –26. C. E. Clifton, Antonie van Leeuwenhoek (A. J. Kluyver, Jubilee Volume), **12,** 186, 1947. – 27. C. B. van Niel and E. H. Anderson, J. Cell. and Comp. Physiol. **17,** 49, 1941. – 28. R. J. Winzler, Science **99,** 327, 1944. – 29. J. M. Reiner, J. Gen. Physiol. **30,** 367, 1947. – 30. M. Stephenson and L. H. Stickland, Biochem J. **27,** 1528, 1933. – 31. M. J. Pinsky and J. L. Stokes, J. Bact. (Proceedings) 127, 1951. – 32. R. Y. Stanier, J. Bact. **54,** 339, 1947.

(Organisch Chemisch Instituut T.N.O., Utrecht).

INVESTIGATIONS ON ORGANIC FUNGICIDES

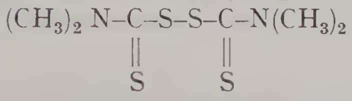
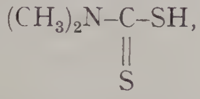
VI. HISTIDINE AS AN ANTAGONIST OF TETRAMETHYLTHIURAM DISULPHIDE (T.M.T.D.) AND RELATED COMPOUNDS[1]

by

A. KAARS SIJPESTEIJN and G. J. M. VAN DER KERK

(Received November 14, 1951).

INTRODUCTION.

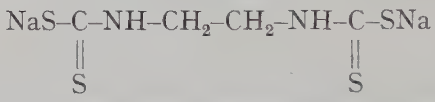
Dithiocarbamates and their oxidation products, the thiuram sulphides, have found wide application in the protection of plants against fungal attack. Of the latter group tetramethylthiuram disulphide, T.M.T.D.,  $(CH_3)_2N\text{–}\underset{\underset{S}{\|}}{C}\text{–}S\text{–}S\text{–}\underset{\underset{S}{\|}}{C}\text{–}N(CH_3)_2$ is a well-known example, whereas the zinc and iron salts of dimethyldithiocarbamic acid,  $(CH_3)_2N\text{–}\underset{\underset{S}{\|}}{C}\text{–}SH$, are economically important representatives of the first group.

Although several suggestions have been made regarding the mode of action of these compounds, convincing evidence is still lacking. HORSFALL (5) connected the antifungal activity of dithiocarbamates and thiuram sulphides with their ability to precipitate metal ions essential to fungal growth. It has, however, been shown recently that different concentrations of Fe, Zn, Cu, Mn and Mo cannot counteract the fungitoxicity of T.M.T.D. towards *Aspergillus niger* (MANTEN, KLÖPPING and VAN DER KERK (10)).

Of a great many organic compounds, mainly sulphur compounds, a subdivision into four specificity groups with different antifungal spectrum was made by Klöpping (6) [1]). Those which under the test conditions are characterized by a relatively high toxicity to *Botrytis cinerea* and *Penicillium italicum* as compared with *Aspergillus niger*, while *Rhizopus nigricans* occupies an intermediate position, are comprised in group I. The lower thiuram disulphides, tetramethylthiuram monosulphide and the lower dithiocarbamates (*e.g.* Na, Fe and Zn dimethyldithiocarbamates) belong to this group. A common mode of action of these compounds was expected by Klöpping on account of this similarity in antifungal spectrum. On the other hand antifungal substances showing a relatively slight toxicity towards *Rh. nigricans* as compared with *B. cinerea* and *P.italicum* and an intermediate activity toward s*A.niger*, were placed in group II. Representatives of this group are disodium ethylenebisdithiocarbamate ("dithane")

---

[1] I: Antonie van Leeuwenhoek **16**, 45, 1950; II: Antonie van Leeuwenhoek **16**, 282, 1950; III: Antonie van Leeuwenhoek **17**, 58, 1951; IV: Rec. trav. chim. **70**, 917, 1951; V: Rec. trav. chim **70**, 949, 1951.



$$\mathrm{NaS{-}\underset{\underset{\displaystyle S}{\|}}{C}{-}NH{-}CH_2{-}CH_2{-}NH{-}\underset{\underset{\displaystyle S}{\|}}{C}{-}SNa}$$

and homologous bisdithiocarbamates and the corresponding diisothiocyanates (*cf.* Klöpping and van der Kerk (8)). Compounds with a high activity towards *B.cinerea* and *P.italicum*, but a low activity towards the two other test moulds are comprised in group III, whereas substances with equal toxicity towards all four moulds were placed in group IV.

It was shown by Klöpping for representatives of group III and IV that the minimal amount required for marked suppression of respiration of *A. niger* and *P.italicum* was about the same as that necessary to inhibit growth. This suggests strongly that growth inhibition by compounds from these groups is due to their interference with respiratory enzymes. A different result was obtained with representatives of group I (sodium dimethyldithiocarbamate) and group II (dithane and tetramethylene diisothiocyanate). Growth of both test moulds was inhibited by concentrations far below those required for inhibition of respiration. Thus the primary action of these compounds on growing moulds is obviously exerted on other than respiratory enzymes.

For the thiuram sulphides and the dimethyldithiocarbamates a possible clue with regard to their mode of action was found in the observation that on a complex medium like peptone-glucose agar or malt agar the toxicity of T.M.T.D. towards *A.niger* was considerably reduced as compared with that on a medium with sugar as the sole organic nutrient (10).

---

[1]) To be published in Rec. trav. chim. in the near future.

This suggested amongst many other possibilities the presence in the complex media mentioned of an antagonist of T.M.T.D. not contained in the simple sugar medium. Consequently, it seemed probable that the most sensitive enzyme system interfered with by T.M.T.D. would be one involved in the synthesis or in the further metabolism of the T.M.T.D.-antagonizing compound.

In the present investigation our first object was a comparison of the toxicity of T.M.T.D. towards four moulds on different media and at different pH values. Next, the nature of the supposed antagonist was elucidated and its effect on the toxicity of T.M.T.D. and chemically related compounds was tested.

FUNGITOXICITY OF T.M.T.D. ON MEDIA OF DIFFERENT COMPOSITION.

Methods.

Botrytis cinerea Pers., *Penicillium italicum* Wehmer, *Aspergillus niger* van Tieghem and *Rhizopus nigricans* Ehrenberg were used as test objects.

As a minimal medium, later referred to as "glucose agar", was chosen: glucose 1 %, $(NH_4)_2SO_4$ 0.1 %, K_2HPO_4 0.25 %, KH_2PO_4 0.25 %, $MgSO_4.7H_2O$ 0.05 %, NaCl 0.05 %, washed agar 2 %, in tap water.

The complex medium used ("casamino acids agar") consisted of this glucose agar additioned with 1 % of an acid hydrolysate of casein (Difco casamino acids, containing 10 % total N). Preliminary experiments had shown that the toxicity of T.M.T.D. was still further reduced in casamino acids-glucose agar than in peptone-glucose agar or malt agar. A further advantage of the use of the hydrolysate is, that the amino acids are available as such to the moulds and that the approximate amounts in which they are present are known.

To achieve optimal growth aneurin (0.2 γ/ml) and biotin (0.002 γ/ml) were always added to the "glucose agar" as well as to the "casamino acids agar" when *P. italicum* or *A.niger* was used.

The toxicity of T.M.T.D. was tested on both media at 6 different pH values between *ca.* 7.2 and 4.0, using the roll-culture method described by MANTEN, KLÖPPING and VAN DER KERK (9). Small bottles containing 1.5 ml of nutrient medium were autoclaved; 0.03 ml of a solution of T.M.T.D. in acetone[1]) and a drop of a heavy spore suspension (containing *ca.* 6000 spores obtained from a malt agar culture) were added to the agar when still at *ca.* 50°. Subsequently the bottles were cooled while being mechanically turned along their longitudinal axis. The growth factors were added together with the inoculum. The pH was adjusted before autoclaving and the exact values were recorded electrometrically in the solid agar after sterilisation.

The range of concentrations of T.M.T.D. in the medium was usually 0; 0.05; 0.1; 0.2; 0.5; 1; 2; 5; 10 p.p.m. Series were always made in duplicate. After 2 and 3 days incubation at 24° C. the minimal concentration of T.M.T.D. at which no growth had occurred was read (minimal inhibitory concentration).

Results.

In Fig. 1 the results are shown. For all four moulds the toxicity of T.M.T.D. was reduced by the presence of 1 % casamino acids. The differences between the toxicity of T.M.T.D. on both media decreased as the pH decreased.

In control bottles (without T.M.T.D.) growth on casamino acids agar was, of course, more abundant than on glucose agar, but growth on neither medium was influenced appreciably by the pH.

A.niger shows an inversion of the T.M.T.D. effect on glucose agar at pH values above *ca.* 6.5, *i.e.* while there was no growth at a certain range of concentrations of T.M.T.D., growth was observed again at higher concentrations. Total inhibition occurred once more at further increase of the T.M.T.D. concentration (Plate I). We shall return to this below.

Allowance must be made for slight experimental variations, which may result in a small parallel shift of the lines separating the growth area from the area of total inhibition. For that reason values somewhat differing from those in Fig. 1 will occasionally be found in the following. These differences may be considered non-essential for the interpretation of our results.

The observations summarized in Fig. 1 supported the view that the casamino acids might contain a compound able to antagonize the action of T.M.T.D.

---

[1]) The resulting acetone concentration in the medium (2%) proved to be harmless.

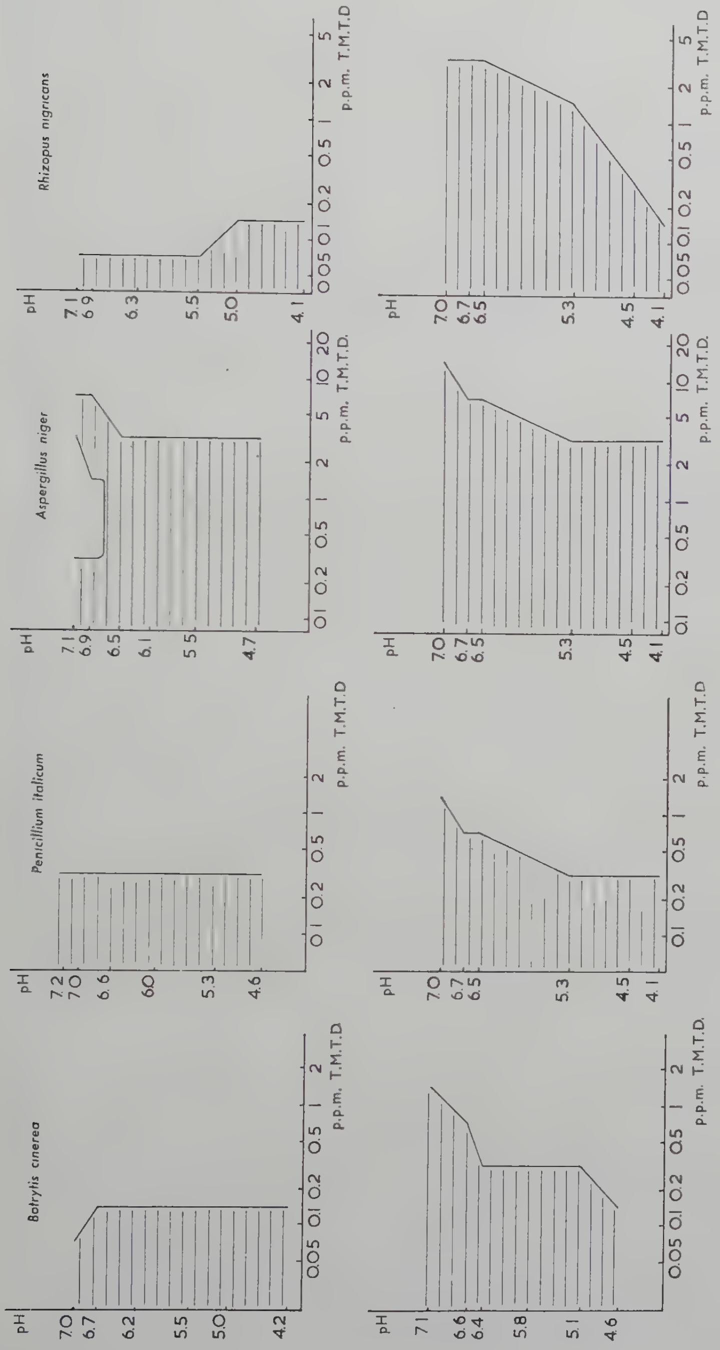


Fig. 1.

Toxicity of T.M.T.D. towards four mould species at various pH values.

Upper row: on glucose agar. Lower row: on glucose agar containing 1% casamino acids. Growth occurred in the shaded areas. Incubation period 3 days; for *Rh. nigricans* 2 days.

IDENTIFICATION OF THE ANTAGONIST OF T.M.T.D. CONTAINED IN CASEIN HYDROLYSATE.

Methods.

The auxanographic technique introduced by BEIJERINCK (2) in 1889 was found very useful in screening the active compound (*cf.* PONTECORVO (12)). Auxanograms were made on the minimal medium (glucose agar). For each experiment a series of agar plates was prepared containing increasing amounts of T.M.T.D.: 0; 0.05; 0.1; 0.2 p.p.m. *etc.* A concentrated solution of T.M.T.D. in acetone, the spore suspension and, for *A.niger* and *P.italicum*, aneurin and biotin were added to the autoclaved medium when still liquid, and plates were poured. The amount of spores per ml nutrient medium was the same as in the roll-culture test mentioned above. On each plate 4 to 6 Pyrex-glass cups were placed and 0.1 ml of a solution of the compound or mixture to be tested was pipetted into the corresponding cups on every plate. Thus 4 to 6 compounds or mixtures could be tested at a time. Since the influence of casamino acids on the toxicity of T.M.T.D. was largest at high pH values, the pH of the medium was so adjusted as to be between 6.8 and 7.0 after autoclaving. The test solutions used in the cups were sterilized by steaming, and their pH adjusted to *ca.* 6.8 with bromothymol blue as an internal indicator.

The antagonizing effect of casamino acids on T.M.T.D. might be due not only to its content of amino acids but also to purines, pyrimidines or growth factors possibly present. Therefore, a series of different mixtures containing these substances was tested on their antagonizing action as well.

The composition of some of the mixtures used in the cups is given below.

18 amino acids.

A solution of a mixture of the following 18 amino acids in the concentration in which they are reported to be represented in casein (BLOCK (3)): L-arginine (420 mg), L-histidine (270 mg), L-lysine (800 mg), L-tyrosine (690 mg), DL-tryptophane (280 mg), L-phenylalanine (520 mg), L-cysteine (30 mg) (used instead of cystine), DL-methionine (700 mg), DL-threonine (820 mg), L-leucine (1000 mg), L-isoleucine (650 mg), L-valine (700 mg), glycine (60 mg), L-alanine (280 mg), DL-serine (1500 mg), L-glutamic acid (2420 mg), L-aspartic acid (630 mg), DL-proline (1600 mg). Where the L-form was not available the required amount was replaced by the double amount of the DL-form.

Except in the first experiment a solution of this mixture in water was always applied at a concentration of $3\frac{1}{2}\%$ total L-amino acids.

9 growth factors.

A solution containing aneurin (10 γ/ml), riboflavin (10 γ/ml), pyridoxine (100 γ/ml), nicotinamide (10 γ/ml), inositol (250 γ/ml), *p*-amino benzoic acid (30 γ/ml), Ca-pantothenate (10 γ/ml), biotin (0.1 γ/ml), folic acid (2 γ/ml).

4 purines + 2 pyrimidines.

A solution containing 10 γ/ml of each of the following components: adenine, xanthin, hypoxanthin, guanine, thymin and uracil.

Results.

a. Experiments with *A.niger*.

A series of plates containing increasing amounts of T.M.T.D. was inoculated with *A.niger*. Aneurin and biotin were omitted from the basal medium in the first two experiments. The following mixtures were added to corresponding cups: (A) 5% casamino acids; (B) 5% vitamin-free casamino acids (Difco); (C) the mixture of 9 growth factors. After 2 days growth was noticeable throughout the basal medium in plates containing 0.5 p.p.m. or less T.M.T.D. On the plates containing 1, 2 and 5 p.p.m. growth was seen around the cups A and B only. Thus in the presence of casamino acids T.M.T.D. was here about 10 times less effective than in their absence. The 9 growth factors, though greatly stimulating normal growth, were unable to counteract T.M.T.D., this substance being equally toxic in their absence as in their presence.

In the second experiment the test solutions were: (A) 3.5% casamino acids; (B) 3.5% casamino acids + 0.03% L-tryptophane; (C) the mixture of 18 amino acids; (D) the mixture of 4 purines and 2 pyrimidines. As tryptophane is known to be destroyed during acid hydrolysis of casein, the effect of its addition to casamino acids was tested. After 2 days the contents of cups A, B and C still gave a growth response at 10 times the maximum concentration of T.M.T.D. which allowed growth in the basal medium. Solution D did not counteract T.M.T.D.

In Table 1 the main results of these two experiments are summarized.

Table 1.

Effect of various additions to the culture medium on the toxicity of T.M.T.D. towards *A.niger*. pH 6.8.

	Growth after 2 days						
p.p.m. T.M.T.D.	0	0.2	0.5	1	2	5	10
Contents of the cups:							
5% vitamin-free casamino acids	+	+	+	±	±	—	—
3½% casamino acids	+ +	+ +	+ +	—	±	—	—
3½% casamino acids + 0.03% tryptophane	+ +	+ +	+ +	—	—	—	—
18 amino acids	+	+	+	+	+	+	—
9 growth factors	+ +	+ +	+	—	—	—	—
4 purines + 2 pyrimidines	±	±	±	—	—	—	—
growth on basal medium	±	±	±	—	—	—	—

From the foregoing it appears that the synthetic mixture of 18 amino acids is as effective as the casamino acids in antagonizing T.M.T.D. The next step was, therefore, an analysis of the action of each of its 18 components.

In order to eliminate possible effects of individual amino acids on normal growth as much as possible, the action of 18 mixtures of 17 amino acids, in which each of the amino acids was left out in turn, was examined instead of the action of every amino acid separately. The concentration of each component was the same as in the "18 amino acids" mixture. In this and subsequent experiments with *A.niger* biotin and aneurin were included in the basal medium to approach optimal growth conditions. The action of the various "17 amino acids" mixtures was compared with that of the "18 amino acids" mixture at increasing concentrations of T.M.T.D. On the control plates without T.M.T.D. growth was homogeneous irrespective of the presence or absence of the test solutions, which demonstrates the adequacy of the aneurin and biotin addition to the basal medium. All but one of the "17 amino acids" mixtures showed the same effect on T.M.T.D.-toxicity as the "18 amino acids" mixture: there was a clear response at 1, 2 and 5 p.p.m. T.M.T.D., while in the basal medium growth was inhibited at these concentrations. Only the mixture lacking histidine showed no response at these concentrations.

These observations suggested strongly that histidine at a pH of about 6.8 is an active antagonist of T.M.T.D. and that it is responsible, to a great extent at least, for the antagonistic action on T.M.T.D.-toxicity of casamino acids, peptone, malt extract *etc.* Its concentration in the 18-amino acids solution was 0.095 %, or 2.7 % of the total L-amino acids.

Further evidence was obtained from the next experiment in which the action was compared of (A) 0.1 % L-histidine, (B) the mixture of 17 amino acids ($3\frac{1}{2}$ % total L-amino acids) lacking histidine, (C) the mixture of 18 amino acids ($3\frac{1}{2}$ % total L-amino acids containing 0.1 % L-histidine in the solution), and (D) 2 % L-histidine.

The results of this experiment are summarized in Table 2; Plate II shows a photograph of the plates after 3 days incubation.

Table 2.

Effect of various additions to the culture medium on the toxicity of T.M.T.D. towards *A.niger*. pH 6.8.

	Growth after 2 days							Growth after 3 days						
p.p.m. T.M.T.D.	0	0.5	1	2	5	10	20	0	0.5	1	2	5	10	20
Contents of the cups:														
A 0.1% L-histidine	+	+	+	+	—	—	—	++	++	++	+	—	±	—
B 17 amino acids (no histidine)	+	+	—	—	—	—	—	++	++	++	—	—	±	—
C 18 amino acids	+	+	+	+	+	—	—	++	++	++	++	+	±	—
D 2% L-histidine	+	+	+	+	+	—	—	++	++	++	++	++	+	—
basal medium + aneurin and biotin	+	+	—	—	—	—	—	++	++	—	—	—	±	—

After two days incubation homogeneous growth was observed on the control plate as well as on the plates with 0.5 p.p.m. or less T.M.T.D. At 1 and 2 p.p.m. T.M.T.D. growth response was seen around cups A, C and D; at 5 p.p.m. around cups C and D only. No growth was seen around cup B at these concentrations.

From these results it may be concluded that the antagonizing effect at pH 6.8 of the casein hydrolysate on the activity of T.M.T.D. is indeed due to its content of L-histidine.

As mentioned above *A.niger* shows an inversion of the T.M.T.D. effect at pH values of the medium exceeding *ca.* 6.5. This phenomenon became visible after 3 days incubation. While on the basal medium no growth occurred at 1, 2 and 5 p.p.m. T.M.T.D., colonies became visible again throughout the agar at 10 p.p.m. T.M.T.D. Though this growth occurred irrespective of the presence of cups with test solutions, it became more abundant in the presence of 2% histidine, "17 amino acids" (lacking histidine) or "18 amino acids". Thus there is no special antagonizing action of histidine at this concentration. On plates containing more T.M.T.D. than the amount which evokes the inversion, no growth was ever observed under the test conditions in the basal medium or around any of the test solutions mentioned. Consequently, the addition of histidine has the effect of overcoming the inhibition caused by T.M.T.D. at concentrations lower than those at which inversion becomes manifest.

b. Experiments with *B.cinerea*, *P.italicum* and *Rh.nigricans*.

The suitability of histidine as an antagonist of T.M.T.D. was checked for these moulds by using the following test solutions in the cups: (A) 0.1% L-histidine; (B) "17 amino acids" (3½% total L-amino acids) lacking histidine; (C) "18 amino acids" (3½% total L-amino acids) containing 0.1% L-histidine; (D) 2% L-histidine.

In Table 3 the results of the experiments with *B.cinerea* and *P.italicum* are summarized. For these moulds an inversion of the T.M.T.D. effect did not occur in this experiment. In later

Table 3.
Effect of various additions to the culture medium on the toxicity of T.M.T.D. towards *B.cinerea* and *P. italicum*. pH 6.8.

	B.cinerea					*P.italicum*				
	Growth after 3 days									
p.p.m. T.M.T.D.	0	0.05	0.1	0.2	0.5	0	0.1	0.2	0.5	1
Contents of the cups:										
A 0.1% L-histidine	+	+	+	—	—	+	+	+	—	—
B 17 amino acids (no histidine)	+	+	—	—	—	++	+	—	—	—
C 18 amino acids	+	+	+	—	—	++	++	+	—	—
D 2% L-histidine	+	+	+	+	—	+	+	+	+	—
Basal medium [1]	+	+	—	—	—	+	+	—	—	—

[1] In the case of *P.italicum* the basal medium contained biotin and aneurin.

experiments this phenomenon was, however, observed occasionally.

On the control plates without T.M.T.D. growth of *B.cinerea* was not influenced by the test solutions, in contrast to *P.italicum* where the mixtures B and C caused a slight growth stimulation.

As in the case of *A.niger* the T.M.T.D. susceptibility of these two moulds shows a clear response to histidine, whereas the mixture of 17 amino acids lacking histidine did not have any effect.

For *Rh.nigricans* the situation was more complicated. On glucose agar 0.1 and 1 % histidine could shift the minimal inhibitory concentration from 0.5 to 5 p.p.m. T.M.T.D. Growth was, however, poor on glucose agar. It appeared to become as good as on the "17-" or "18-amino acids" mixtures by addition of 1 or 2 % DL-proline to the cups. Proline alone had no effect on the toxicity of T.M.T.D. Proline additioned with 0.1 % or 1 % histidine could again shift the minimal inhibitory concentration from 0.5 to 5 p.p.m. T.M.T.D. Histidine in a concentration of 2 % was slightly inhibitory, also on the control plate without T.M.T.D. The 18 amino acids mixture gave the same picture as 0.1 % histidine + proline. In the presence of the "17 amino acids" mixture there was no growth response at 0.5 p.p.m., but an inversion of the T.M.T.D. effect was visible at 1 and 2 p.p.m.

The specificity of histidine towards T.M.T.D. was further checked for *P.italicum* by comparing the antagonistic effect of a 2 % solution of each of the 17 other L-amino acids with that of 2 % L-histidine. Where only the DL-form was available, a 4 % solution of the DL-form was added to the cups. Owing to insufficient solubility DL-tryptophane was tested as a 1 % solution, DL-methionine as a 2 % solution, L-isoleucine as a 1.5 % solution, whereas L-tyrosine was tested as a saturated solution (solubility 0.05 g/100 ml). The addition of histidine could raise the minimal inhibitory concentration of T.M.T.D. considerably. No other amino acid showed a comparable effect at the pH value used (6.8), but two amino acids, L-cysteine (2 %) and DL-methionine (2 %), were able to counteract the T.M.T.D.-inhibition to a small extent.

These 2 amino acids did not influence growth on the plate without T.M.T.D. Their minor T.M.T.D.-antagonizing activity may perhaps account for the fact that the mixture of 18 amino acids sometimes shows a slightly better antagonistic activity than 0.1 % histidine alone.

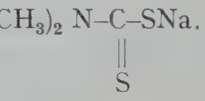
Similar results were obtained with *A.niger* for the amino acids tested (serine, proline, tryptophane, cysteine, methionine); 2% L-cysteine and 2% DL-methionine could raise the minimal inhibitory concentration of T.M.T.D. Whereas the effect of methionine was very restricted, cysteine gave a greater and qualitatively somewhat different response. In a future communication the cysteine effect will be described in detail.

EFFECT OF HISTIDINE ON THE TOXICITY OF SOME COMPOUNDS STRUCTURALLY RELATED TO T.M.T.D.

The effect of histidine on the fungitoxicity of the following compounds, structurally more or less related to T.M.T.D., was investigated: sodium dimethyldithiocarbamate, tetramethylthiuram monosulphide (T.M.T.M.), disodium ethylenebisdithiocarbamate ("dithane"), tetramethylene diisothiocyanate [1]).

Auxanograms were made at pH 6.8-7.0, as described above and the following test solutions were applied again: (A) 0.1% L-histidine (B) "17 amino acids" (3½% total L-amino acids) lacking histidine; (C) "18 amino acids" (3½% total L-amino acids) containing 0.1% L-histidine in the solution; (D) 2% L-histidine.

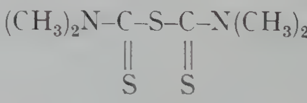
a) Sodium dimethyldithiocarbamate,



$(CH_3)_2N{-}\underset{\displaystyle S}{\overset{}{\underset{\|}{C}}}{-}SNa.$

Experiments with *B.cinerea*, *P.italicum* and *A. niger* showed qualitatively the same results as with T.M.T.D., as might be expected for this reduction product of T.M.T.D.

b) Tetramethylthiuram monosulphide,



$(CH_3)_2N{-}\underset{\displaystyle S}{\underset{\|}{C}}{-}S{-}\underset{\displaystyle S}{\underset{\|}{C}}{-}N(CH_3)_2.$

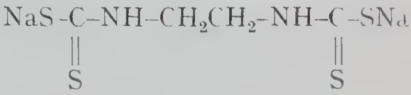
Growth of *B.cinerea* and *P.italicum* was inhibited at 0.5 p.p.m., that of *A.niger* at 1 p.p.m.; an inversion of the T.M.T.M. effect was observed for all three moulds at 50–100 p.p.m. For *A.niger* the first zone of inhibition was completely overcome by "18 amino acids", as well as by 0.1% or 2% L-histidine added to the cups.

---

[1])The chemical preparation and the antifungal activity of these compounds are described in the communications IV and V of this series (7, 8).

For the other two moulds only the addition of 2 % histidine seemed sufficient to obtain the same effect. There was a slight antagonistic action of the mixture of 17 amino acids towards T.M.T.M. for all three moulds.

c) Disodium ethylenebisdithiocarbamate,



$NaS-\underset{\underset{S}{\|}}{C}-NH-CH_2CH_2-NH-\underset{\underset{S}{\|}}{C}-SNa$

No antagonistic action was observed of histidine (0.1 %, 1 %), "17 amino acids", "18 amino acids", "9 growth factors" or a mixture of adenine, xanthine, uracil, thymine (100 γ of each/ml) added to cups on plates inoculated with *A.niger*.

d) Tetramethylene diisothiocyanate, $S=C=N-(CH_2)_4-N=C=S$.

Histidine (0.1 %, 2 %), "17 amino acids", "18 amino acids" could not counteract the toxicity of this compound towards *A.niger*.

As pointed out in the introduction of this paper the lower dithiocarbamates belong to another specificity group than the bisdithiocarbamates and the diisothiocyanates (Group I and II respectively). It was suggested that compounds belonging to different specificity groups might have a different mode of antifungal action. The observed ineffectiveness of histidine against representatives of Group II as compared with its striking effect against compounds belonging to Group I lends strong support to the correctness of this view.

THE FUNGITOXICITY OF T.M.T.D. AND T.M.T.M. IN RELATION TO THE COMBINED EFFECT OF THE pH OF THE MEDIUM AND OF THE AMOUNT OF HISTIDINE PRESENT.

The experiments recorded above were all carried out at pH 6.8–7.0. The influence of the pH of the medium and of the amount of histidine present on the antagonistic action of this substance towards T.M.T.D. and related compounds was studied by the roll-culture method.

Experiments with T. M. T. D.

Six series of bottles were prepared containing glucose agar with the following pH values respectively: 7.0, 6.8, 6.5, 6.1, 5.1 and 4.1. After addition of increasing amounts of T.M.T.D. to each of the series, the bottles were inoculated with spores of *P.italicum*. A second set was treated in the same way, but each bottle received after autoclaving in addition 0.05 ml of a sterile solution of 1.2% L-histidine of the proper pH-value to give a final concentration of 0.04% L-histidine in the medium. The result is shown in Fig. 2.

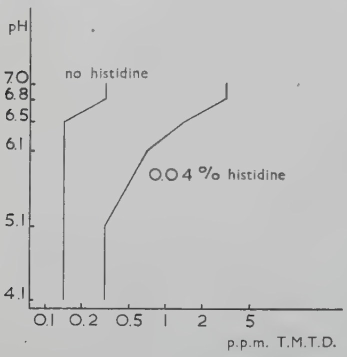


Fig. 2.
Effect of pH of the medium (glucose agar) on the concentrations of T.M.T.D. preventing growth of *P.italicum*, in the absence and in the presence of L-histidine.
Incubation period 3 days.

Histidine appeared to antagonize T.M.T.D. over the whole pH range investigated, though it seemed more effective at the higher pH values. The latter point was confirmed by the results of the following experiment.

Bottles were prepared with 0, 0.001, 0.003, 0.01, 0.03, 0.1 and 0.3% histidine in the glucose medium. For one series the pH was adjusted to 6.9, for a second series to 5.2. Increasing amounts of T.M.T.D. were added, and the bottles were incubated after inoculation with *P.italicum*. From the results (Fig. 3) two conclusions can be drawn. In the first place it appears that there is a close parallelism between the fungitoxicity of T.M.T.D. and the amount of histidine in the medium; in fact the observed interaction of these substances suggests a competitive relation. Secondly, the histidine effect on T.M.T.D. toxicity appears smaller at pH 5.2 than at pH 6.9.

The same experiment was carried out with *B.cinerea* with essentially the same result.

In the following experiment the influence of the pH of the medium on the antagonistic action of histidine towards T.M.T.D. was investigated for *A.niger*. Bottles were prepared with glucose agar of the pH values 7.0, 6.8, 6.1, 5.1, 4.6. After addition of increasing amounts of T.M.T.D. to the sterile medium, the bottles were inoculated with spores of *A.niger*. A second series was treated in the same way, but received in addition 0.04% L-histidine. On the glucose medium the result was the same as obtained earlier (*cf.* Plate I). As shown by Plate III, an inversion of the T.M.T.D. effect was visible at pH 7.0 and 6.8, separating a first zone of growth suppression from a second zone of inhibition. The addition of histidine results in the complete disappearance of the first zone of growth inhibition. The T.M.T.D. concentrations causing this first zone of inhibition at high pH values are, however, completely non-toxic at pH 6.1 and lower. It thus seems that two ways exist to protect the most sensitive cell systems against the action of T.M.T.D.: firstly, at high pH values, by adding L-histidine, secondly, by merely lowering the pH of the culture medium. In the second zone of growth inhibition the addition of 0.04% of histidine was without any effect over the whole range of pH values tested. In another experiment 0.1, 0.3 and 1% of histidine were incorporated in the medium at pH 7.0 and 5.7 respectively. Exactly the same results were obtained even with these high histidine concentrations.

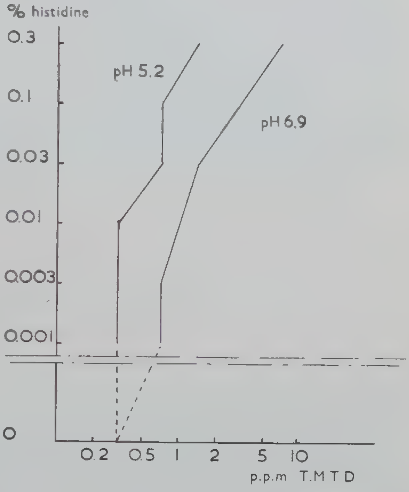


Fig. 3.
Influence of the amount of histidine on the concentration of T.M.T.D. preventing growth of *P.italicum* at pH 5.2 and pH 6.9.
Incubation period 3 days; medium: glucose agar.

A comparison of Fig. 2 with Fig. 1 gives once more evidence that the antagonistic action of casamino acids is due to its content of histidine.

Experiments with T. M. T. M.

On glucose agar an inversion of the T.M.T.M. (tetramethylthiuram monosulphide) toxicity was found for *P.italicum* over the whole range of pH values tested (Table 4).

In another experiment it was shown that at pH 6.8 addition of 0.01% histidine to the medium is sufficient to completely over-

Table 4.

Toxicity of T.M.T.M. towards *P.italicum* on glucose agar at various pH values. Incubation period 4 days.

	p.p.m. T.M.T.M.											
pH	0	0.1	0.2	0.5	1	2	5	10	20	50	100	200
7.1	+	+	+	—	—	—	—	—	—	+	+	—
6.8	+	+	+	—	—	—	—	—	—	+	+	—
6.4	+	+	+	—	—	—	—	—	+	+	—	—
6.1	+	+	+	—	—	—	—	—	+	+	—	—
5.4	+	+	+	—	—	—	—	—	+	+	—	—
4.7	+	+	+	—	—	—	—	—	+	+	—	—

come the first zone of inhibition. At pH 5.6 0.1 % histidine was required to overcome this inhibition, whereas at pH 4.8 even as much as 1 % histidine failed to produce this effect. In the latter case the minimal inhibitory concentration of T.M.T.M. was shifted only from 1 to 5 p.p.m. by the addition of 0.3 or 1 % histidine to the medium; there was no growth at 5, 10, 20 and 50 p.p.m. and slight growth (inversion) at 100 p.p.m. T.M.T.M.

Similar results were obtained with *B.cinerea*. On glucose agar an inversion of the T.M.T.M. effect occurred only at pH values higher than *ca.* 5.4 (Table 5).

Table 5.

Toxicity of T.M.T.M. towards *B.cinerea* on glucose agar at various pH values. Incubation period 5 days.

	p.p.m. T.M.T.M.											
pH	0	0.1	0.2	0.5	1	2	5	10	20	50	100	200
7.0	+	+	+	—	—	—	—	—	+	+	—	—
6.6	+	+	+	—	—	—	—	—	+	+	—	—
6.3	+	+	+	—	—	—	—	+	+	—	—	—
5.6	+	+	+	—	—	—	—	+	+	—	—	—
5.4	+	+	+	—	—	—	—	—	—	—	—	—
4.6	+	+	+	—	—	—	—	—	—	—	—	—

At pH 7.0 the first zone of inhibition could be overcome by 0.03 % histidine, at pH 5.5 it was almost completely overcome by 1 % histidine, whereas at pH 4.7 the latter amount could shift

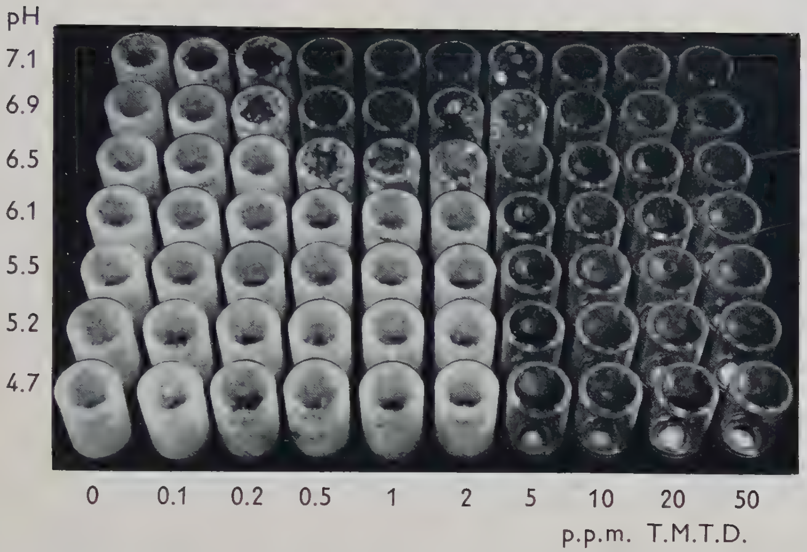


Plate I.

Growth of *A.niger* on glucose agar at various pH values in the presence of increasing amounts of T.M.T.D.

Incubation period 4 days.

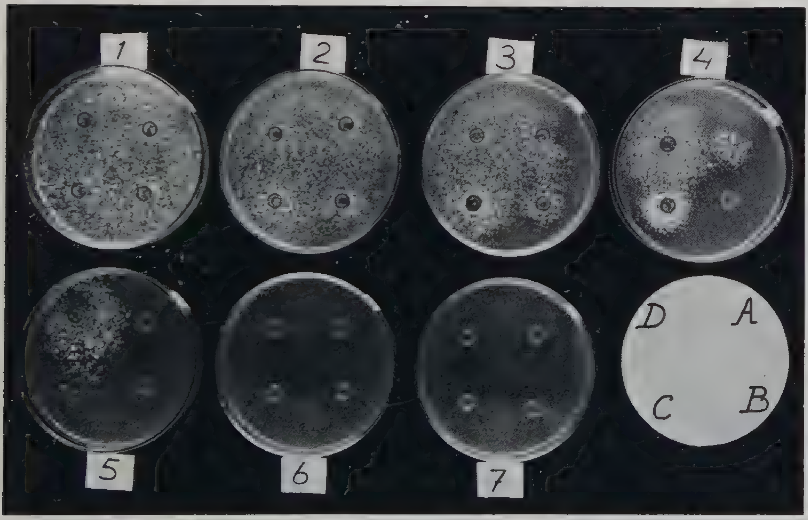


Plate II.

Auxanograms showing the effect of various additions to the culture medium on the toxicity of T.M.T.D. towards *A.niger* after 3 days of incubation.

T.M.T.D. concentrations in plates 1—7: 0, 0.5, 1, 2, 5, 10 and 20 p.p.m. respectively. Contents of the cups: *A* 0.1% L-histidine, *B* 17 amino acids lacking histidine, *C* 18 amino acids, *D* 2% L-histidine. pH 6.8.

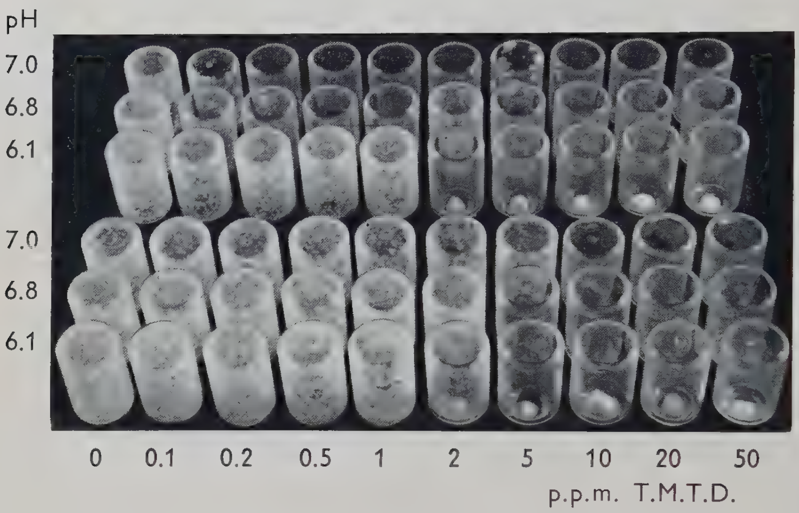


Plate III.

Influence of the addition of 0.04% of L-histidine on the toxicity of T.M.T.D. towards *A.niger* at pH 7.0, 6.8 and 6.1.

Upper half: without histidine. Lower half: with 0.04% L-histidine. Incubation period 3 days; medium: glucose agar.

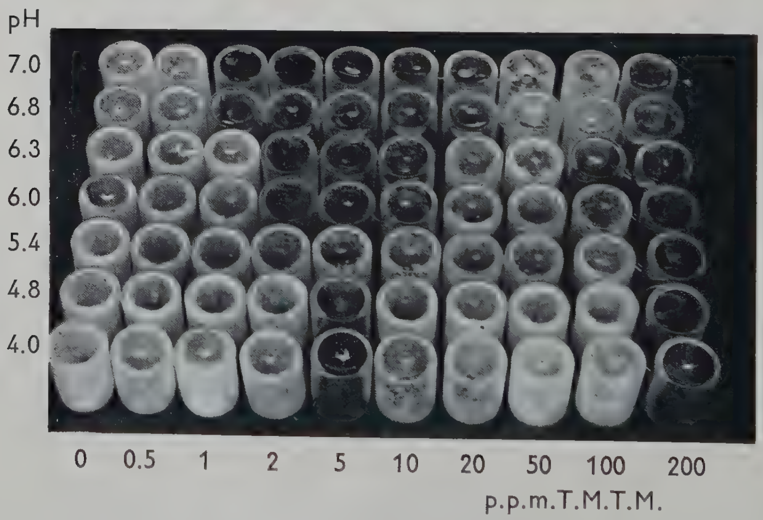


Plate IV.

Toxicity of T.M.T.M. towards *A.niger* on glucose agar at various pH values. Incubation period 4 days.

the minimal inhibitory concentration only from 0.5 to 1 p.p.m. T.M.T.M.

Thus for both moulds the effect of the addition of histidine to the medium diminishes with decreasing pH. Corresponding results were reported above for T.M.T.D.

The effect of T.M.T.M. on growth of *A.niger* at different pH values (Plate IV) differs somewhat from that of *P.italicum* and *B.cinerea*, but resembles the response of *A.niger* to T.M.T.D. The first zone of inhibition observed at high pH values can again be completely overcome by histidine. At pH 7.0 as little as 0.01 % histidine is sufficient to achieve this effect.

Thus, the reaction of the three different mould species on T.M.T.D. and T.M.T.M. appears to show individual features, but the antagonistic action of histidine towards T.M.T.D. and T.M.T.M. inhibition at high pH values is common to all of them. The inversion phenomenon, though incomprehensible as yet, appears to be closely connected with the histidine effect.

EFFECT OF SODIUM DIMETHYLDITHIOCARBAMATE AND OF T.M.T.D. ON SPORE GERMINATION AND ON MYCELIAL GROWTH.

The experiments mentioned above all refer to the effect of toxicants on mycelial growth preceded by spore germination. The following experiment was set up to investigate whether the lowest quantities that inhibit spore germination are also inhibitory to mycelial growth.

To this end twenty three 125 ml conical flasks were filled with 20 ml of glucose medium of pH 6.8 (the minimal medium mentioned before, omitting the agar). After autoclaving biotin and aneurin were added and all flasks were inoculated with *P.italicum* spores (*ca.* 15,000 per ml). One series of nine flasks immediately received quantities of sodium dimethyldithiocarbamate ranging from 0.02 to 10 p.p.m. All twenty three flasks were incubated at 24° C. in the shaking machine (shaking speed 80 strokes per minute). After 24 hr. a first indication of mycelial growth could be observed macroscopically in the second series (twelve flasks), the pH being still unchanged. These flasks then received quantities of the fungicide ranging from 0.02 to 100 p.p.m. The two remaining flasks served as controls. The results after a total incubation period of 5 days can be seen from Table 6.

Table 6.

Effect of sodium dimethyldithiocarbamate on spore germination and on mycelial growth of *P.italicum* at pH 6.8.

Na-dimethyldithio-carbamate, p.p.m.	Growth after 5 days												
	0	0.02	0.05	0.1	0.2	0.5	1	2	5	10	20	50	100
Fungicide added at start	++	++	++	+	—	—	—	—	—	+			
Fungicide added after 24 hr. incubation	++	++	++	++	++	+	+	+	++	++	++	+	—

It is evident that in the case of spore germination the minimal inhibitory concentration of sodium dimethyldithiocarbamate is far less than in the case of mycelial growth. A remarkable feature in this and other experiments with shaking cultures of *P.italicum* is that an inversion effect of the dimethyldithiocarbamate and of T.M.T.D. is always observed in contrast to agar cultures of this mould where this effect is absent or only weakly indicated. Mycelial growth was slightly depressed at 0.5, 1 and 2 p.p.m. This indicates that below the minimal inhibitory concentration for mycelial growth the fungicide apparently is not completely harmless to this process either.

The effect of the simultaneous presence of histidine was investigated in an experiment similar to the previous one by including two additional series of flasks receiving 0.1 % of histidine at the moment of the addition of the fungicide. A control flask without fungicide was included in each series. The results are summarized in Table 7.

From Table 7 it can be seen firstly that histidine counteracts the growth inhibition caused by sodium dimethyldithiocarbamate, but only below the concentration of the fungicide at which inversion becomes manifest. Secondly, the antagonizing effect of histidine seems to be restricted to the action of the dimethyldithiocarbamate on the process of spore germination, although a slightly beneficial effect on mycelial growth can also be noted.

Similar results were obtained with T.M.T.D. Thus it seems firmly established that the main effect of the fungicides of this type in the concentration range investigated is exerted on the process of spore germination; the growing mycelium under these circumstances responds only with a slight growth retardation, mycelial growth being not completely inhibited.

Table 7.

Effect of sodium dimethyldithiocarbamate on mycelial growth of *P.italicum* in the absence and in the presence of histidine. pH 6.8.

Na-dimethyldithiocarbamate, p.p.m.	Growth after 4 days										
	0	0.05	0.1	0.2	0.5	1	2	5	10	20	50
Fungicide added at start	++	+	—	—	—	—	—	—	+	—	—
Fungicide + 0.1% histidine added at start	++	++	++	++	++	++	+	++	++	—	—
Fungicide added after 24 hr. incubation	++	++	++	+	±	+	+	++	++	—	—
Fungicide + 0.1% histidine added after 24 hr. incubation	++	++	++	++	++	++	++	++	++	—	—

DISCUSSION.

The present investigations have shown convincibly for four test moulds that the inhibition of growth caused by small amounts of T.M.T.D. on a glucose-mineral salts medium is counteracted by L-histidine. Thus the histidine content of "casamino acids" appears to be responsible for the observed difference between the fungitoxicity of T.M.T.D. on a simple glucose-mineral salts medium and on media containing this complex nutrient.

Sodium dimethyldithiocarbamate and T.M.T.M. for which the same mode of action was suggested as for T.M.T.D. (KLÖPPING (6)) proved indeed to be also antagonized by histidine. In contrast hereto histidine did not show any antagonizing effect on the chemically closely related bisdithiocarbamate "dithane" and on tetramethylene diisothiocyanate. This result again might be expected since the latter two compounds after KLÖPPING belong to another specificity group than the thiuram sulphides and the dimethyldithiocarbamates.

It is reasonable to assume that T.M.T.D. (and most probably also T.M.T.M. and the dimethyldithiocarbamates) either interfere with the biosynthesis of histidine or with some essential reaction for which histidine is required. Though the compitative character of the histidine-T.M.T.D. interaction might be in favour of the second possibility, the experimental data presented in this paper do not allow to draw any definite conclusion.

An inversion of the toxicity of T.M.T.D., T.M.T.M. and sodium dimethyldithiocarbamate was observed with certain moulds. The inversion phenomenon has been observed before in spore-germination tests (*cf.* DIMOND, HORSFALL, HEUBERGER and STODDARD (4), MONTGOMERY and SHAW (11), BARRATT and HORSFALL (1)), but its dependence on pH and composition of the medium was made clear by the present investigation. The cause of the inversion phenomenon is not yet properly understood[1]). It is obvious, however, that there is a relation with the histidine antagonism in so far as histidine is effective only at concentrations of the toxicant below that at which visible inversion occurs.

At low pH values histidine is far less effective than at high pH values. An explanation of this fact cannot yet be given, but in connection herewith the following may be remarked. Notable individual differences exist between the response towards T.M.T.D., and especially towards T.M.T.M., of the test organisms used at low pH values. With *A.niger* the first zone of inhibition can be overcome at pH values higher than 6 by the addition of histidine, whereas at pH values below 6 this occurs also without histidine. In contrast hereto *B.cinerea* and *P.italicum* show the same response to histidine at high pH values, but with decreasing pH values the antagonistic effect of histidine gradually disappears. These observations may suggest that at low pH values T.M.T.D. and T.M.T.M. interfere with a system different from the one in which histidine is involved.

The minimal inhibitory concentration of T.M.T.D. and of sodium dimethyldithiocarbamate was smaller when spores were used than when applied on growing mycelium. Those concentrations which inhibit development of spores, and at which histidine has an antagonistic effect, do not seem to prevent growth of the mycelium.

---

[1]) In a recent communication (H. EAGLE, J. Bact. **62, 663, 1951** an interesting suggestion has been forwarded to explain a similar phenomenon observed in the action of penicillin on certain bacteria.

Moreover, histidine cannot alter the concentrations of these toxicants at which mycelial growth is prevented. It is evident that the inversion phenomenon is restricted to the process of spore germination and that, consequently, any future explanation of this interesting phenomenon must be sought in this primary phase of fungal development.

Regarding the primary mode of action of T.M.T.D. and related substances one may conclude that the most sensitive enzyme system interfered with by these compounds has a specific function in the process of spore germination. This again might be an explanation for the fact that fungi are exceptionally sensitive to these compounds as compared with bacteria and phanerogamae.

Summary.

L-Histidine was found to antagonize the toxicity of the fungicides tetramethylthiuram disulphide (T.M.T.D.), tetramethylthiuram monosulphide (T.M.T.M.) and sodium dimethyldithiocarbamate towards *Botrytis cinerea*, *Penicillium italicum*, *Aspergillus niger* and, in the cases studied, towards *Rhizopus nigricans*. The antagonism showed a competitive character but as yet no definite conclusion could be drawn whether these compounds interfere with histidine synthesis or rather with some essential reaction in which histidine is involved. At high pH values histidine proved to be more effective than at low pH values. The histidine effect is restricted to the process of spore germination; this process is far more sensitive to the toxicants mentioned than mycelial growth. The inhibition of the latter process is not counteracted by histidine.

With increasing concentration of the fungicides an inversion of their toxic effect was observed for certain of the test moulds under specified conditions. This inversion phenomenon too is connected with the process of spore germination and shows a close relationship with the histidine antagonism in so far as histidine is only effective at concentrations below those at which inversion becomes manifest.

Histidine cannot antagonize the action of the fungicides disodium ethylenebisdithiocarbamate ("dithane") and tetramethylene diisothiocyanate. This supports the view that the mode of antifungal action of these compounds is different from that of the chemically related lower thiuram sulphides and dimethyldithiocarbamates.

References.

1. R. W. Barratt and J. G. Horsfall, Connecticut Agr. Expt. Sta. Bull. **508**, 1, 1947. – 2. M. W. Beijerinck, Arch. néerl. Sci. **23**, 367, 1889. – 3. R. J. Block, Adv. Protein Chem. **2**, 119, 1945. – 4. A. E. Dimond, J. G. Horsfall, J. W. Heuberger and E. M. Stoddard, Connecticut Agr. Expt. Sta. Bull. **451**, 635, 1941. – 5. J. G. Horsfall, Fungicides and their action. Waltham, Mass. U.S.A., 1945. – 6. H. L. Klöpping, Chemical constitution and antifungal action of sulphur compounds. Thesis Utrecht, 1951. – 7. H. L. Klöpping and G. J. M. van der Kerk, Rec. Trav. Chim. **70**, 917, 1951. – 8. H. L. Klöpping and G. J. M. van der Kerk, Rec. Trav. Chim. **70**, 949, 1951. – 9. A. Manten, H. L. Klöpping and G. J. M. van der Kerk, Antonie van Leeuwenhoek **16**, 282, 1950. – 10. A. Manten, H. L. Klöpping and G. J. M. van der Kerk, Antonie van Leeuwenhoek **17**, 58, 1951. – 11. H. B. S. Montgomery and H. Shaw, Nature **151**, 333, 1943. – 12. G. Pontecorvo, J. gen. Microbiol. **3**, 122, 1949.

(National Institute of Public Health, Utrecht).

SILVER IMPREGNATION OF SMALLPOX ELEMENTARY BODIES AFTER TREATMENT WITH XYLOL

by

R. GISPEN

(Received October 24, 1951).

In 1926 MOROSOW (Zentralbl. f. Bakt. I, **100**,385,1926) introduced a modification of FONTANA's silver impregnation as a method for staining Paschen bodies. The modification may give excellent results under carefully controlled conditions. Unfortunately, success depends on the material examined and the mode of treating, that vary considerably in the practice of smallpox diagnosis. Moreover, a thin film is required, which should be extracted in distilled water before fixation. Much of the material is lost during this treatment. Preliminary fixation or an increase of film thickness interferes with the aim of extraction.

In our experience however, it appeared that prolonged extraction with xylol, preceded by fixation with RUGE's solution, can be executed to make the smear invariably suited for silver impregnation. In this way, the film thickness can be increased considerably and the treatment with distilled water omitted without interfering with the reaction of virus bodies on mordant and silver stain. The independence from fat content and from thickness of smears eliminates an inconvenience, perhaps an obstacle with microscopic examination for Paschen bodies.

The complex silver solution according to MOROSOW's prescription cannot be easily reproduced. Constant good results can be obtained when the ammoniacal silver precipitate is solved by adding a minimal quantity of 1 % ammonia and the balance is restored at a bluish opalescence by one drop of 10 % silver nitrate.

Solutions required.
Solution A (fixative): glacial acetic acid, 1 ml; formalin, 2 ml; distilled water, 100 ml.
Xylol in a cylinder glass with a ground in glass stopper.
Solution B (mordant): carbolic acid, 1 ml; tannin, 5 g, distilled water, 100 ml.
Ammonia: 25% solution.
Ammonia: 1% solution.
Silver nitrate: 10% solution.

Preparation of the complex silver solution.
Wash a clean Erlenmeyer flask with distilled water. Bring a loopfull of 25% ammonia solution into the remaining drop of water. Add 0.5 ml of 10% silver nitrate. Add 20 ml of distilled water. Add 1% ammonia, drop by drop, until the ammoniacal silver precipitate redissolves suddenly at one drop (about 1.7 ml). One drop of 10% silver nitrate is added, bringing about a bluish opalescence being just visible with transillumination. The solution cannot be stored.

Procedure.
Scrapings from the base of lesions are smeared on a clean slide and dried in air. Treat with solution A for 1 minute. Rinse in tap water; dry in air. Place the slide on end in a wide mouth bottle with xylol for 5 minutes; evaporate in air. Treat with solution B and heat until steam rises, for 1 minute. Rinse thoroughly with tap water and wash again with distilled water. Prepare the complex silver solution according to the above prescription with carefully cleaned glassware and add it immediately. Heat until steam rises for 2 minutes. Rinse in tap water and dry in air. Mount in liquid paraffin.

(Department of Bacteriology and Experimental Pathology, Institute for Preventive Medicine, Leiden, Holland).

ISOLATION OF SMALLPOX VIRUS FROM THE NASOPHARYNX OF PATIENTS WITH VARIOLA SINE ERUPTIONE

by

J. D. VERLINDE and **H. A. E. VAN TONGEREN**

(Received October 24, 1951).

During a local outbreak of smallpox in a town in the Netherlands, which will be described elsewhere, the clinical differential diagnosis between smallpox and chickenpox appeared to be difficult in many of the patients, because of a partial immunity induced by mass vaccination and revaccination four years previously. The laboratory diagnosis, however, proved highly valuable, and for this reason the contents of papules and vesicles, and crusts, were examined by three different methods:

1. examination of smears for elementary bodies after staining with the methods of Paschen, modified by Van Rooijen and Illingworth, Gutstein, Herzberg, and of Morosow modified by Gispen (2);
2. complement fixation reaction with vesicle fluid or extract from crusts, and vaccinia hyperimmune serum;
3. isolation of virus by inoculation of the chorioallantoic membrane of the chick-embryo.

In the direct examination for elementary bodies in smears, the method of Morosow-Gispen proved most satisfactory: typical elementary bodies were found in 82% of the cases of variola, and in 50% of vaccinia vesicles examined. The variola and vaccinia elementary bodies could be distinguished from those of chickenpox by their size and number.

The complement fixation reaction was positive in 82% of the virologically proven cases of variola, and in 31% of the virologically proven cases of vaccinia. A positive reaction was also found in one in 25 cases of chickenpox.

The virological diagnosis by egg inoculation proved to be the most valuable diagnostic procedure, as this was positive in 100% of the cases of smallpox, vaccinia, and cowpox examined. In contrast to the other procedures, this method permits an immediate differentiation between the viruses of variola, vaccinia, and cowpox by naked-eye and microscopic examination of the infected egg membrane (3). No smallpox lesions were observed on the skin and in the mouth and the throat.

With only a few exceptions, the cases of smallpox could be explained by contact with a smallpox patient. The few exceptions, however, initiated an investigation into the occurrence of clinically unrecognizable smallpox infections, which has been suspected also in other epidemics (LE BOURDELLÈS, LESAFFRE and ROGEZ (1)).

In order to get an answer to the question whether the carrier state would occur in man, not suffering from clinically recognizable smallpox, nasopharyngeal secretions of 13 persons chosen from the isolated contacts were examined for the presence of variola virus over a number of successive days. These secretions were obtained either by swabbing or by gargling with saline. The swabs were washed in 0.5 to 1 ml of saline, and the washings or garglings were inoculated onto the chorioallantoic membrane of the chick-embryo after treatment with 1000 units of penicillin and 2.5 mg of streptomycin per ml.

In the nasopharyngeal secretions of two of the contacts examined, variola virus was shown to be present ¹).

1. A 17-year old maid-servant in a family in which two adults contracted smallpox, one on April 13, the second on May 1. The girl had been vaccinated and revaccinated twice, the date of the last revaccination being May 4. On May 12 transient prodromal symptoms appeared, but no virus was isolated from the throat.

2, 3. Two males, 35 and 36 years of age, who had had repeated contact from April 26 to May 11 with a man, who contracted smallpox on May 5, and died on May 16. Both of them had been revaccinated on May 5. No virus could be recovered from the throat of either of the contacts between the 9th and the 13th day after the first, and between the 3rd and the 7th day after the last contact.

4. A woman, 35 years of age, who had washed a patient suffering from variola (eruptive stage) on May 6. She was revaccinated on May 7. Prodromal symptoms appeared on May 17, which lasted for two days. No smallpox

---

¹) We are indebted to Drs E. M. AUSEMS and TH. J. WETERMAN of the Municipal Health Service, Tilburg, and to Dr P. J. V. VAN WESEMAEL, Inspector of Public Health, Breda, who kindly supplied the data of these contacts.

lesions were observed on the skin, and in the mouth and the throat. Throat-washings were examined on May 20 and 29. From the first specimen, taken on the 14th day after the contact, variola virus was isolated. The first egg-passage was doubtful, the second was obviously positive, and in sections of the chorioallantoic membrane of the third passage GUARNIERI bodies were found. The membrane lesions were characteristic for variola, and different from those produced by vaccinia and cowpox virus. On May 29 no virus was recovered from the throat.

5. A woman, 27 years of age, had been in contact on May 13 and 14 with a patient, who fell ill on May 9, and showed a rash on May 13. She was vaccinated in childhood, and revaccinated on April 30. In throat-washings collected on May 29, no virus could be shown.

6. Woman, 54 years of age, whose husband showed prodromal symptoms on May 11, and a rash on May 14. She had been vaccinated in childhood and revaccinated on May 4. On May 29 no virus was recovered from the throat.

7. Woman, 32 years of age, whose husband contracted smallpox on May 9. She had been vaccinated in childhood, and revaccinated on May 4. No virus was isolated from the throat on May 29.

8, 9, 10, 11, 12, 13. Six contacts of a non-vaccinated girl, 18 years of age, who contracted smallpox on May 25 (rash on May 28). All but one of the contacts had been vaccinated or revaccinated in May, and the contact had occurred either during the prodromal stage or on the first day of the rash. The throat-washings were examined daily from the 8th to the 18th day after the contact.

In one of them, i.e. a 20-year old girl, who had visited the patient on the first and the second day of the prodromal stage, variola virus could be isolated from the throat on the 18th day after the contact. In this case also the first egg-passage was doubtful, but the second and third passage were positive, and GUARNIERI bodies could be shown in sections of the egg-membrane. A repeated virological examination of the throat 7 days later yielded a negative result.

It is noteworthy, that the last case, a non-vaccinated girl, has not contracted smallpox, not even prodromal symptoms, although she was controlled clinically very carefully. Yet, a subclinical infection has to be assumed, since vaccinia virus neutralizing antibodies were shown in the blood one month after the contact by means of the intradermal rabbit neutralization test. The serum proved to neutralize 80 minimal infecting doses of vaccinia virus per ml. Unfortunately no blood sample was taken immediately after the contact, so that it remains unanswered whether or not antibodies were already present. This, however, seems most unlikely, since this girl has neither been vaccinated, nor has lived in a country where smallpox is endemic. Moreover, she was born after the alastrim epidemic which occurred in the Netherlands in 1929.

Evidence has been obtained from this investigation, that variola virus may be present in nasopharyngeal secretions during the prodromal stage, which may occur even when the individual has been vaccinated early in the incubation period, and in nasopharyngeal secretions of the healthy, non-vaccinated contact, who may become a virus carrier without contracting the disease, although this seems to be extremely rare. In this case the non-vaccinated contact has been infected by a smallpox patient during the first two days of the prodromal stage. The carrier state does not seem to last longer than one or perhaps a few days.

The few cases of smallpox, which could not be explained by contact with a patient suffering from smallpox, may be explained by contact either with an abortive case, which may have shown only prodromal symptoms because of a certain degree of immunity, or by a healthy virus carrier. Large scale investigations on this subject are necessary in order to elucidate the frequency and the duration of the carrier state.

Summary.

In 2 out of 13 contacts of smallpox patients, variola virus was recovered from nasopharyngeal secretions on the 14th and the 18th day after the last contact. One of them was vaccinated, and showed transient prodromal symptoms, the other, who had never been vaccinated did not show signs of illness. Evidence has been obtained from this investigation, that a few cases of smallpox during a local epidemic, which could not be explained by contact with a smallpox patient, may be explained by contact with a virus carrier.

References.

1. B. le Bourdellès, V. Lesaffre et Ch. Rogez, Off. Int. Hyg. Publ. **38,** 449, 1946. – 2. R. Gispen, Antonie van Leeuwenhoek **18,** 107, 1952. – 3. J. D. Verlinde and H. A. E. van Tongeren, to be published.

(From the Laboratory for Tropical Hygiene of the State University, Leiden).

EXPERIMENTS WITH AN APPARATUS FOR LYOPHILE DESICCATION OF MICRO-ORGANISMS

by

HENDRIK L. WOLFF

(Received November 21, 1951).

At the symposion on freeze-drying recently held in London a number of questions were brought forward with regard to the desiccation of bacteria. We are in a position to answer some of these questions for the procedure followed in our laboratory, but as our apparatus differs in rather important points from those that are used in England, it is uncertain in how far our conclusions may be applied to the latter. Nevertheless it seems worth while to publish our data.

Since 1947 we subject all the bacterial strains and viruses we work with in this laboratory, to lyophile desiccation in order to preserve them for future use. The apparatus we constructed for this purpose was tested in various ways in order to find out how it should be handled to yield the best results.

APPARATUS.

We work with a mechanical oil-rotation pump (Cenco Hyvac) by means of which a vacuum of 10^{-3} to 10^{-2} mm Hg can be obtained (1). For the collecting of the moisture that is set free we use a vessel cooled by liquid air (2). Our preference for liquid air as refrigerating fluid is due to the circumstance that we can obtain this substance at a low price. [1] The pressure is controlled with a MacLeod manometer (3). In order to connect the ampoules or flasks with the apparatus we use two kinds of connecting vessels: 4b are simple glass bottles with one or more side tubes into which the ampoules or flasks are attached; 4a is a metal container with a larger number of side tubes (fig. 1).

---

[1] For this as well as for many valuable indications we are greatly indebted to the Staff of the Kamerlingh Onnes Laboratory.

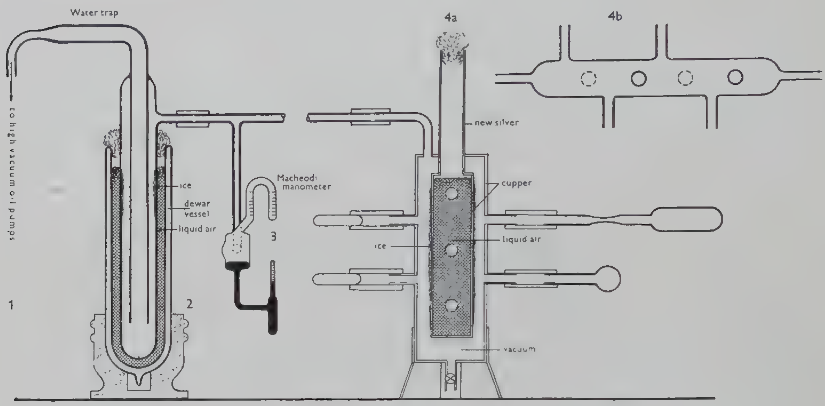


Fig. 1 Diagrammatic representation of the apparatus for lyophile desiccation.

1. high vacuum oil pump
2. water trap (second stage)
3. manometer (MacLeod)
4a. water trap connecting vessel (Histrix aquamretinens)
4b. connecting vessel

General remarks.

It is advisable to restrict the use of rubber exhausting tube for the connection of the various parts as much as possible, and the moisture catcher must be large enough to collect all the water that is set free. As the latter is frozen into a kind of snow, this procedure requires a vessel of comparatively large size. The ampoules should be made of glass that melts at a low temperature. They are sealed by heating in vacuo; the necessary skill has to be gained by experience.

The pressure should be under control continually in order to obviate disagreable surprises, e.g. cracks in the glass parts, etc. Although every pressure below 1.4 mm Hg will keep the mass that we wish to desiccate, in a frozen condition, it is advisable to choose a pressure between 0.01 and 0.4 mm Hg. A further decrease of the pressure offers, in general, no advantages. The air-replacing capacity of the pump system, the temperature, which can be regulated by means of the irradiating heat, and the surface extension of the material we wish to desiccate, are the factors which determine the rate of the process of drying. The specific material we wish to desiccate, and also a too great thickness of the layer in which the latter is subjected to the drying process, may act unfavourably. In order to avoid pressure fluctuations the total contents of the vacuum system should not be taken too small.

THE FREEZING OF THE MATERIAL TO BE DESICCATED.

Various methods of freezing were compared. As the results with other strains agree very well with those attained with a strain of *E. coli* (93), we shall confine ourselves to the results obtained with the latter. The bacteria were suspended in 0.25 ml skimmed milk. The frozen material was always dried in the same way. The dry material was suspended in 25 ml broth. The values in the tables apply to 1/20 ml (1 standard drop) of this suspension. The number of surviving bacteria was counted by means of roll cultures, made according to the method of JULIUS (2). Determinations were all carried out in duplo and the mean value was taken up.

Table 1.

The number of bacteria surviving various methods of freezing.

Treatment	Surviving bacteria				Average
Original culture (control)	2017	2007	1980	1972	1994
Slowly frozen to –10°C.	42	41	39	38	40
Rapidly frozen to –10°C.	337	317	315	310	320
Slowly frozen to –35°C.	278	278	276	272	276
Rapidly frozen to –35°C.	1881	1843	1843	1824	1848

Table 1 clearly shows that the line along which the freezing is carried out very markedly influences the final result. Rapid freezing proves to furnish much better results than slow freezing. The fact that slow freezing to —35°C. proved better than rapid freezing to —10°C. is in our opinion due to the circumstance that in the former case the temperature falls more rapidly to the critic range. We have also studied the action of temperatures below —35°C. but with no or hardly better results than those with freezing to —35°C. So we have in the main confined ourselves to rapid freezing at —35°C.

Technically the freezing is carried out in the following manner. The small glass container in which the fluid is enclosed, is immersed in ethanol or ethyl-glycol at —35°C., the container being rapidly rotated round its axis. Within a few seconds a thin layer of frozen substance is formed against the wall of the container. When ampoules are used the freezing offers no difficulty. When, however, flasks containing a larger quantity of liquid are used, care should be taken that a sufficiently large volume of cooling fluid is available. Moreover, it is in this case advisable to use a cooling fluid of a somewhat lower temperature. The frozen layer should not be too thick as in that case the freezing process is retarded and this causes a decrease in the number of surviving bacteria. The desiccation, moreover, becomes more difficult.

The figures given in Table 1 refer to material that was first frozen and then dried. We have tried to ascertain how the temperature as such acts on the bacteria, using the same material as in Table 1, rapidly frozen to —35°C. Slow thawing was effected by keeping the ampoules at + 4°C. in the air-conducting tubes of a refrigerator; rapid thawing was obtained by placing the ampoules in a water bath at 37°C.

Table 2.

Number of bacteria surviving various methods of thawing.

Treatment	Number of surviving bacteria				Average
Original culture (control)	2017	2007	1980	1972	1994
Thawed slowly	327	324	323	320	323
Thawed rapidly	936	918	918	900	918

It appeared that the process of thawing influenced strongly the number of surviving bacteria. Here again slow thawing was more detrimental than rapid thawing.

In order to obtain further information as to the effects of freezing and thawing we have transferred the frozen material from —35°C. to —10°C. and vice versa.

Table 3 clearly shows that a transfer of the frozen mass from — 35°C. to — 10°C. and vice versa entails death of many bacteria. These results were admittedly a surprise to us. So we have extended our experiments in order to find out whether a transfer to — 180°C. would have a similar effect. Table 4 gives the results.

Table 3.

Number of bacteria surviving a change in freezing temperatures.

Treatment	Number of surviving bacteria				Average
Kept at –35°C., then dried (control)	1734	1717	1717	1700	1717
a. transferred from –35°C. to –10°C. then dried	664	659	658	654	659
b. transferred from –35°C. to –10°C. and back to –35°C., then dried	558	556	551	549	554
c. treatment b twice repeated	218	216	213	211	215
d. treatment b thrice repeated	151	150	139	139	145

Table 4.

Number of bacteria surviving a transfer to very low temperatures.

Treatment	Number of surviving bacteria				Average
Kept at –35°C., then dried (control)	1224	1206	1206	1188	1206
a. transferred from –35°C. to –180°C., then dried	1242	1242	1206	1206	1224
b. transferred from –35°C. to –180°C., and back to –35°C., then dried	1242	1224	1188	1170	1206
c. treatment b twice repeated	1224	1224	1188	1170	1201
d. treatment c thrice repeated	1260	1242	1188	1188	1219

Results of subsequent experiments are taken up in Table 5.

Table 5.

Number of bacteria surviving a storage at –8°C. during periods of various length directly before drying.

Treatment	Number of surviving bacteria				Average
Transferred from –35°C. to –8°C.	564	559	558	554	559
½ hour at –8°C.	521	519	506	501	512
1 hour at –8°C.	478	465	461	451	464
20 hours at –8°C.	218	201	198	197	203

The turning point lies apparently in the neighbourhood of —35°C. Above this temperature the noxious effect is always noticeable; below hardly or not all at. The period in which the frozen mass is kept at temperatures above —35°C., appears to have a marked effect.

All these experiments were carried out in skimmed milk as suspension liquid. We have tried also other media (serum, serum-broth, ascitic broth, whey, ordinary broth, broth-gelatin). None of them gave such good results as skimmed milk. However, it is worth noting that the results obtained with these media were in the main the same as those obtained with skimmed milk.

These results led us to the following conclusions:

a. The freezing must be effected as rapidly as possible by cooling to a temperature of —35°C. or lower;

b. It is desirable to keep the temperature during the process of desiccation at —35°C., or, if this proves impossible, to make the desiccation as rapid as possible.

c. The frozen material can be preserved for some time without much deterioration at temperatures of —35°C. or lower.

THE DESICCATION OF THE FROZEN MATERIAL.

In order to investigate how our strains could best be desiccated we have used the apparatus described above. It appeared to be very difficult to comply with our first conclusion of keeping the material during the desiccation at a temperature of —35°C., at least if we wished to dry it within a reasonable length of time. It proved difficult to determine exactly the temperature of the drying mass. We estimated the latter at —20° to —25°C. The temperature of the glass wall was measured by means of a thermo-element. By limiting the amount of irradiation it is easy to obtain a lower temperature, but in that case the rate of desiccation is too slow. We have tried to meet this difficulty by decreasing the pressure in our desiccator, but without satisfactory results.

Several points demanded an explanation:

a. Does the final result depend on the manner of desiccation?

b. How long does it take before the material is dry?

c. Is there a simple means to determine this moment?

Like in the case of the freezing we have tried to ascertain whether the rate of the process of desiccation acts on the final result. Without succes, however. Up to now we have been unable to dry at temperatures of —35°C. and lower, at least within a period of 24 hours. Therefore we had to dry at higher temperatures and keeping in mind the experiments reported on, it is not to be wondered at that here as well the results proved influenced by the length of time during which the material was subjected to various temperatures.

When the apparatus was used in the form of 4a, we obtained somewhat better results. This arrangement, however, works less economically, as it requires a larger volume of liquid air. The insertion of a second water trap (fig. 1; 2) in the chain does not make much difference. We do it in order to safeguard our pump.

How do we determine the stage at which the desiccation may be regarded as complete? In Table 6 the results of our experiments are taken up.

Table 6.

Number of bacteria surviving periods of desiccation of various length.

Length of period of desiccation	Number of surviving bacteria									
	Tested at the end of the desiccation period				Av.	Tested 24 hours later				Av.
$\frac{1}{4}$ hour	634	612	608	591	611	219	212	199	198	207
$\frac{1}{2}$ hour	794	791	775	761	780	254	249	241	237	245
$\frac{3}{4}$ hour (thawing point)	1164	1131	1122	1116	1133	642	631	629	603	626
1 hour	1530	1513	1489	1455	1497	1472	1467	1449	1419	1452
$1\frac{1}{2}$ hour	1530	1530	1513	1472	1511	1547	1531	1530	1516	1531
2 hour	1547	1513	1496	1472	1507	1531	1530	1498	1480	1510
3 hour	1530	1513	1472	1472	1497	1550	1547	1513	1472	1520

We did not succeed in finding an exact measure for the critical stage of desiccation. Fortunately the figures given in Table 6 indicate that it does not matter very much how far the desiccation is continued. The moment that admits the most exact determination is that at which the temperature rises above 0°C., i.e. the moment when the amount of heat withdrawn by the evaporation becomes too small to counterbalance the amount of irradiating heat (thawing point). When the ampoule is sealed before the thawing point has been reached, marked deterioration is noted. By sealing at the thawing point itself the number of surviving bacteria is lower than that reached half an hour later; after a longer interval the figures remain constant. Similar values were obtained when instead of skimmed milk another medium was used for the desiccation.

The next point to be settled was the sealing of the ampoules. Some investigators fill them with dry inert gas (N) before sealing; some seal in vacuo, while others admit dry air. The advantage of a gas filling is that then the sealing will be easier. When the sealing is performed in vacuo, one always runs the risk that pressure cracks may be formed; the latter sometimes appear after a considerable lapse of time. With more experience this risk gradually decreases: in fact, we ourselves have but rarely been troubled by these cracks.

We have counted the number of surviving bacteria in ampoules that had been stored for periods varying in length. The results are given in table 7.

Table 7.

Number of bacteria surviving a various length of storage.

Length of storage	Number of bacteria				Average
1 day	1550	1547	1513	1472	1520
2 days	1532	1531	1502	1467	1508
7 days	1547	1513	1498	1449	1502
14 days	1532	1502	1498	1496	1507
31 days	1513	1511	1494	1465	1496
61 days	1539	1520	1499	1482	1510
364 days	1513	1511	1482	1467	1493

Even after a year there proved to be no appreciable deterioration. We regret that we possess no data with regard to ampoules that have been stored for a longer period, although we have dried all our strains since 1947.

As our results are quite satisfactory, we deem it not necessary to admit inert gas, so we have not studied the admission of gases.

With regard to the desiccation we arrived at the following conclusions:

a. As our apparatus does no allow a desiccation of the material at a temperature of —35°C. within a reasonable lapse of time, the question whether the final result is influenced by the length of the drying period remains unanswered. When the desiccation is carried through at a higher temperature, it is obviously advisable to terminate the desiccation as soon as possible. However, even then it remains desirable to keep the temperature low.

b. The desiccation may be brought to a close one half to one hour after the outside surface of the ampoule has reached the thawing point.

c. We leave open whether admission, before sealing, of inert dry gas into the ampoules may be desirable.

Two other problems have been studied in connection with the preceding experiments:

1. Is it possible that the desiccation causes a change in the characters of the bacteria?

We worked originally with strains that had been kept in the laboratory for a considerable length of time, and in these strains we never observed any change. Recently, however, we have discovered that special characters may be lost during desiccation. We have dried *Brucella* strains that possessed a special antigen (6); after desiccation it appeared that this strain, which originally had been "smooth", had become "rough". Other dried *Brucella* strains however, retained their "smoothness". We have tried to find out why one of the strains had become "rough", whereas the other ones remained "smooth" (S). To this end we have studied the action of media varying in constitution, pH and condition (solid or liquid). When the suspensions of bacteria after boiling for two hours in a NaCl solution were tested as to "roughness" (R) we could note the following results:

a.	grown and dried in skimmed milk (10% CO_2). pH 8.4	SRRR
b.	grown and dried in ascitic broth (10% CO_2). pH 7.8	SSRR
c.	grown on broth agar (10% CO_2), dried in skimmed milk. pH 7.8	SSSS
d.	grown on ascitic agar (10% CO_2), dried in skimmed milk. pH 8.4	SSSS
e.	grown on glycerol agar, dried in skimmed milk. pH 7.8	SSSR
f.	grown on broth agar (10% CO_2), dried in skimmed milk. pH 7.4	SRRR
g.	grown on broth agar (10% CO_2), dried in ascitic broth. pH 7.8	RRRR

It appeared that suspensions in skimmed milk of bacteria that had been grown on a solid medium and a pH between 7.8 and 8.4 gave the best results. We wish, however, to make plain that these results for *Brucella* should not be generalized. The smoothness of pneumococci provided with capsule is best preserved when starting from cultures in ascitic broth with a pH of 7.4. With Enterobacteriaceae, especially with Salmonellae, Shigellae and Escherichiae (suspensions in skimmed milk with a pH of 7.4 — 7.8) we experienced no difficulties. Even unstable factors like Vi antigen are well preserved, but it is important to transfer the strains to the ampoules directly after suspending, to freeze them rapidly and to keep them at —35°C until they can be desiccated. The cultures that are to be lyophylized always should be fresh, the inoculation should preferably be carried out 12—24 hours previously. Before as well as after desiccation the characters of the strain should be checked as to the occurrence of changes.

2. Is a cross infection during the process possible? When more than one kind of micro-organisms are to be desiccated simultaneously with the same apparatus, this question is very important. We have studied this problem by means of the apparatus 4b.

After desiccating simultaneously a culture of *Proteus vulgaris* (X. 19) and one of E. *coli*, we have washed the glass connecting vessel (4b) with diluted broth, which subsequently was poured out on Endo and McConkey plates. On the latter a few colonies of X. 19 and also some of *E. coli* developed. It appeared that the infection took place at the moment the ampoules were sealed. The dry powdery contents are easily separated from the wall and may be sucked into the connecting vessel. This points to a current of air, but how does the latter arise? It can not be due to leakages, for these were detected immediately and repared. However we had already noticed that the pressure inside the apparatus fluctuates very markedly (from 0.02 to 0.3 mm Hg), and this, of course, causes currents. In order to eliminate this fluctuation we have increased the capacity of our apparatus and obtained in this way: *a*. that the pressure remained constant, and *b*. that it was no longer possible to grow bacteria from the glass connecting vessel. The risk of cross infection, that previously always had been present, was in this way excluded.

It is worth while to add that cross infections may also be due to another cause, which is especially effective at a higher temperature (circ. —10°C.,) *viz.*, to the explosion of water vapour bubbles (3).

DISCUSSION.

It should be plainly understood that we started this investigation in order to find out whether our apparatus for the desiccation of micro-organisms answers it purpose, and if so, how it should be handled to give the best results. We certainly do not mean to say that our apparatus is better than those used by other investigators, however, we hope that our data will provide a suitable base for future comparison. There are in our opinion, some objections against the method developed by GREAVES (4), in which the material is frozen by rapid evaporation. It is, of course, an advantage that the ampoules are centrifuged during the process of refrigeration. This decreases the risk of cross infections caused by exploding vapour bubbles. Whether this is entirely satisfactory we are unable to judge. Our ampoules are closed by means of a plug of cotton wool (twisted round a wooden tooth pick) immediately after they had been filled. With the apparatus of EDWARDS the ampoules remain for a long time in open communication with the outside air, and this increases the risk of infection. With our method this risk is far smaller. However, our main objection against the method of freezing by means of rapid evaporation is that temperature falls too slowly and that it does not fall low enough (v. supra).

We have as yet no experience with the mixture used by GREAVES e.a. (5), but in the near future we hope to find an opportunity to test the latter.

The apparatus offered for sale by EDWARDS solves in an elegant manner the problem how to desiccate large amounts of infectious material. Our apparatus can in its present form certainly not be used for this purpose.

One of the advantages of our own apparatus is that it can be sterilised entirely by vapour (120°C.). Desinfection by means of alcohol is very inadequate for lyophylized material, as many micro-organisms and viruses are resistant against this treatment. On the whole it is better to use steam.

We do not give the results we obtained with the desiccation of viruses, because the number of quantitative data is still too small. We may point out, however, that the majority of the viruses can be inactivated by simply washing the apparatus with destilled water. We deem sterilization by means of vapour at a temperature of 120°C., however more effective, and it is certainly to be recommended when dangerous virus strains are to be dealt with.

Summary.

Lyophyle desiccation is an extremely valuable method of pre-

serving micro-organisms. The apparatus described above proves satisfactory. The material should be frozen as rapidly as possible at a temperature of —35°C. or lower, and the apparatus should be constructed in such a way that the pressure remains approximatively constant. During the sealing of the ampoules care should be taken that no dry material passes from the latter to the apparatus. A time limit for the desiccation process is hard to fix. When water has condensed on the outside surface of the ampoules, the apparatus is left untouched for about an hour. This brings the total duration of the desiccation as a rule up to 1½ to 2 hours. A volume of 0.25 ml skimmed milk suffices for the preservation of a strain. It is advisable to start from a fresh culture that has been grown on a solid medium and to suspend the required amount in skimmed milk at a pH of 7.4 — 7.8. The suspension should directly be frozen in a thin layer. Before the frozen ampoules are submitted to the desiccation process, they may be kept for some time at a temperature of —35°C.

References.

1. Symposium on freezing and drying. 29–30 June 1951, London. – 2. H. W. Julius, Antonie van Leeuwenhoek **5**, 28, 1938. – 3. Wilson Smith at the Symposium. – 4. R. I. N. Greaves, British Science News **2**, No. 18, 1951. – 5. R. M. Fry at the Symposium. – 6. H. L. Wolff and J. E. Dinger, J. Path. Bact. **63**, 163, 1951.

[Institut National de la Recherche Agronomique, Laboratoire de Physiologie Acoustique, Jouy-en-Josas (Seine et Oise), France].

PHOTOSENSIBILITE DU *BACILLUS CEREUS* var. *ALESTI* AU RAYONNEMENT ULTRAVIOLET DE 2537 Å

par

C. VAGO et **M. C. BUSNEL**

(Reçu le 6 Novembre 1951).

Un grand nombre de bactéries, de virus et de moisissures ont une photosensibilité très grande pour le rayonnement ultraviolet; la détermination du seuil de photoinactivation d'un germe présente donc un grand intéret, d'une part en vue de l'utilisation pratique du rayonnement ultraviolet pour obtenir une stérilisation et d'autre part en vue d'atténuation de la virulence d'une culture pour préparer un vaccin.

Dans le cadre des problèmes de la pathologie entomologique, l'étude des techniques pratiques de désinfection de matériel d'élevage, et celle de la prophylaxie par des souches microbiennes atténuées, donnait à l'emploi des U.V. une place prépondérente.

C'est en raison des deux aspects de ce problème que nous avons étudié la photosensibilité d'un des microorganismes les plus pathogènes pour les insectes, le *Bacillus cereus* var. *Alesti*.

MATÉRIEL ET MÉTHODES DE TRAVAIL.

Nous avons utilisé des lampes à vapeur de mercure, à basse tension et basse pression, de 25 W. émettant principalement sur la longueur d'onde 2537 Å (lampes germicides, type de la Cie. des Lampes).

Les préparations bactériologiques étaient irradiées sous les lampes, à une distance de 5 cm, et recevaient un rayonnement évalué à 2300 milli-W. par cm^2/minute. La température mesurée par thermocouple ne s'est jamais élevée après plus de 5 heures d'irradiation au niveau des cultures de plus de 2° C. au dessus de la température du laboratoire, elle était au maximum de 25° C.

Les temps d'irradiation ont varié de 60 à 900 minutes, avec 30 minutes de différence entre chaque série.

Le *B. cereus* var. *Alesti* utilisé (souche no. 11)[1]) provenait de l'hémolymphe de *Bombyx mori* L. prélevée au stade 3 de la maladie.

Culture sur gélose nutritive (formule standard américaine). Les cultures étaient utilisées après une incubation de 48 heures après formation complète de spores, soit directement par irradiation des boites de Pétri, soit par irradiation des microbes isolés, à sec, obtenus par étalement et frottis de culture sur lame de verre.

Après les irradiations, prélèvement des cultures sur la gélose et les frottis, ensemencement sur gélose nutritive, incubation de 48 heures et contrôle du développement microbien. Pour chaque temps d'irradiation, on a traité 10 cultures et 10 frottis.

Résultats.

Les moyennes des résultats obtenus sur les cultures sur gélose et sur les microorganismes isolés sur lame, sont indiquées dans le tableau suivant:

Temps d'irradiation en minutes	Energie U.V. reçue par cm² en milliwatt	Développement de la culture de contrôle en provenance: des colonies sur gélose	des microbes isolés sur lame
60	138	+++	+++
90	207	+++	+++
120	276	+++	+++
150	345	+++	+++
180	414	+++	+++
210	483	+++	+++
240	552	+++	+++
270	621	+++	+++
300	690	+++	+++
330	759	+++	+++
360	828	+++	+++
390	897	+++	+++
420	966	+++	+

---

[1]) Fédération Internationale des Collections de Cultures-Types. Collection des microorganismes pathogènes aux Invertébrés, Station de Recherches Séricicoles, Alès (Gard), France.

Temps d'irradiation en minutes	Energie U.V. reçue par cm² en milliwatt	Développement de la culture de contrôle en provenance: des colonies sur gélose	des microbes isolés sur lame
450	1035	+++	+
480	1104	+++	—
510	1173	+++	—
540	1242	+++	—
570	1311	+++	—
600	1380	+++	—
630	1449	+++	—
660	1518	++	—
690	1587	+	—
720	1656	+	—
750	1725	+	—
780	1794	—	—
810	1863	—	—
840	1932	—	—
870	2001	—	—
900	2070	—	—

La photoinactivation de *B. cereus* var. *Alesti* se manifeste à partir de 390 minutes (6,30 h.) pour les microbes isolés à sec, et de 660 minutes (11 h.) pour les colonies sur gélose; elle est totale à partir de 480 minutes (8 h.) pour les premiers et de 780 minutes (13 h.) pour les seconds.

On peut donc considérer comme dose léthale d'U.V. pour le *B. cereus* var. *Alesti* irradié en culture, 1863 milli-W/cm², correspondant dans nos essais à 810 minutes d'irradiation (13,30 h.); le microorganisme isolé sur une lame est détruit par 1173 milli-W/cm² soit 510 minutes d'irradiation (8,30 h.) dans les conditions précitées.

Etant donné que les rayons U.V. ont un pouvoir de pénétration extrèmement faible, on peut attribuer à l'épaisseur de la colonie sa plus grande résistance par rapport à celle des microbes isolés.

Ces résultats déterminent la photosensibilité de *B. cereus* var. *Alesti* aux rayons U.V. de 2537 Å; ils permettent d'envisager l'utilisation de ce rayonnement à des fins d'application spécifiques.

(From the Virus Laboratory of the Clinic of Internal Medicine,
University of Leyden).

THE INFLUENCE OF TREATING FERRET INFLUENZA ANTISERA WITH ENZYMES OF CRUDE FILTRATE OF *VIBRIO CHOLERAE* ON THE TITRE OF THE ANTIBODIES [1]

by

J. MULDER, MISS I. DE NOOIJER and L. M. BRANS

(Received November 18, 1951).

Burnet *et al.* (2, 3) discovered that enzyme containing filtrates of *Vibrio cholerae* are capable of eliminating the non-specific inhibition against influenza virus. Since then this procedure was applied in the routine serological examination of influenza virus strains with the aid of the haemagglutination inhibition test (Mulder and van der Veen (5), van der Veen and Mulder (8), Smith and Westwood (7), Chu *et al.* (4), Appleby and Stuart Harris (1)). It became evident that for most strains the crude filtrate, and not the R.D.E. is necessary to neutralize the non-specific inhibition of antisera. The method is only permissible when it can be proved that crude filtrate does not affect the antibodies. Mulder and van der Veen, Chu *et al.* and Appleby and Stuart Harris reported that an appreciable fall in the titre of the antibodies does not occur after treatment with a crude filtrate (strain 4Z) [2]. Smith and Westwood employing the sensitive photo-electric titration method, noted a fall in the titre of the antibody which they attributed to the presence of proteinase in the filtrate. We have gone into this problem once again with the aid of the egg protection test.

---

[1] Financial support was provided by the Institute for Preventive Medicine, Leyden; N. V. Philips Roxane, Weesp; the State Department of Science; the Jan Dekker Fund, and the Curaçao Fund for Preventive Medicine.

[2] Kindly sent to us by Prof. Dr F. M. Burnet.

MATERIALS AND METHODS.

Egg protection test. The strains of influenza virus were grown in the allantoic cavity of embryonated eggs. After incubation for 2 days the allantoic fluid was centrifugated for 10 minutes (3500 revolutions). The M.E.I.D.$_{50}$ was calculated according to REED and MUENCH (6). The egg protection test was performed with twofold dilutions of ferret antiserum. A series of tests was performed with serum that had been treated with potent filtrate (5 parts filtrate added to one part serum). The potency of each filtrate was tested against the strain A-prime Barratt (1947, Eng.), known to be highly susceptible to non-specific inhibition. The pH of each filtrate did not exceed 7.6. The sera were treated for a period of 18 hours at a temperature of 37°C. Subsequently the mixture was heated for one hour at 56°C., so as to destroy any remaining enzyme. In a control test the serum was diluted with physiological saline and underwent the same heatings as the filtrate treated serum. The serum-virus mixture was kept 30 minutes at +2°C., before inoculating the eggs. We used a final dilution of 1000 M.E.I.D.$_{50}$ of the virus and 4 eggs per serum dilution. The protective titres of the antisera were calculated according to REED and MUENCH.

Haemagglutination inhibition test. One day after the egg protection test the haemagglutination inhibition test was done in duplicate with the same treated and untreated sera that were used in the egg protection test. The sera were kept at 2°C. overnight. The micro-method technique according to VAN DER VEEN and MULDER was employed (8).

RESULTS.

Table 1 shows the results of 5 tests. They establish that in the egg protection test it is not possible to demonstrate an appreciable fall in the titre of antibodies due to treatment with enzymes of crude filtrate. The experiment with the B-strain Budapest (1949, Hung.) is particularly interesting The haemagglutination inhibition tests with this strain show a clear reduction in antititre of the treated sera. This must be attributed to the addition of the effect of the non-specific and specific inhibition on the antigen in the untreated antiserum (VAN DER VEEN and MULDER (8)).

Summary.

After treating influenza antisera from ferrets with enzymes of a crude filtrate of *Vibrio cholerae* (pH 7.6) for the purpose of eliminating the non-specific inhibitors it is not possible to detect a significant fall in the titre of the antibodies with the aid of the egg protection test.

Table 1.

Egg protection and haemagglutination inhibition tests with ferret antisera treated and not treated with crude filtrate of *Vibrio cholerae* (strain 4Z).

ANTI SERUM (Ferret)	TITRE				Exp.
	Egg protection test		Haemagglutination inhibition test 1)		
	Treated (chol. filtr. 5 : 1)	Not treated (Saline)	Treated (chol. filtr. 5 : 1)	Not treated (Saline)	
A–Ala (1941, U.S.A.) (A–PR8-type)	110	120	...	...	1
A (1941, Ned.) (A–PR8–type)	305	305	.../5120 2) .../4608	.../5120 2) .../4608	2
A SL (1941, Ned.) (A–PR8–type)	1935	1536	<12/20480 3) <12/18432	1152/32768 3) 1152/32768	3
Bud. (1949, Hung.) (B–type)	34	34	<12/746 3) <12/672	373/2986 3) 373/2688	4
Bud. (1949, Hung.) (B–type)	33	48	<12/746 3) <12/672	56/2389 3) 56/2389	5

1) Done in duplo.

2) Only post-infection ferretserum used.

3) Pre- and post-infection ferretserum used.

References.

1. J. C. Appleby and C. H. Stuart Harris, Brit. J. Exp. Path. **31**, 797, 1950. – 2. F. M. Burnet, J. F. McCrea and J. D. Stone, Brit. J. Exp. Path. **27**, 228, 1946. – 3. F. M. Burnet and J. D. Stone, Austr. J. Exp. Biol. Med. Sc. **25**, 227, 1947. – 4. C. M. Chu, C. H. Andrewes and A. W. Gledhill, Bull. World Hlth. Org. **3**, 187, 1950. – 5. J. Mulder and J. van der Veen, Acta med. brev. Neerl. **16**, 57, 1948. – 6. L. J. Reed and H. Muench, Am. J. Hyg. **27**, 493, 1938. – 7. W. Smith and M. A. Westwood, Brit. J. exp. Path. **30**, 48, 1949. – 8. J. van der Veen and J. Mulder, Studies on the antigenic composition of human influenza virus A strains. Onderz. en meded. Instit. Praev. Geneesk. **6**, 1950, Leiden (p. 20, 7 and 24).

(From the Clinic and Virus Laboratory of the Department of Medicine,
University of Leyden).

STUDIES ON THE INCREASE OF ANTIBODIES IN NASAL SECRETIONS OF HUMAN BEINGS AFTER INTRACUTANEOUS VACCINATION WITH INFLUENZA VIRUS, WITH THE AID OF THE HAEMAGGLUTINATION INHIBITION TECHNIQUE [1]

by

J. MULDER, L. M. BRANS and J. F. Ph. HERS

(Received November 18, 1951).

The work of Fazekaz de St Groth and Donelly (3) has shown the important role which the titre of post-vaccinal respiratory antibodies against influenza virus plays in provoking a protection against subsequent intranasal infection of experimental animals (mice).

Burnet *et al.* (1) and Francis (4) observed that nasal secretions of human beings may contain neutralizing antibodies against influenza virus. The titre of this antibody is low and it rises after natural infection (Francis and Brightman (5)), and also after subcutaneous vaccination (Francis *et al.* (6)). It is therefore certain that these respiratory antibodies are identical with those in the blood serum. Hence Francis in 1941 expressed the view that the outcome of prophylactic vaccination against influenza will depend more on its effect on the titre of the antibodies in the respiratory tract than on that in the blood serum.

Respiratory antibodies against influenza virus in man have as yet been studied on a small scale only. We have endeavoured to develop a rapid method to render possible a comprehensive

---

[1] Financial support was provided by the Institute of Preventive Medicine, Leyden; N. V. Philips Roxane, Weesp; the State Department of Science; the Jan Dekker Fund, and the Curaçao Fund for Preventive Medicine.

study of antibodies against influenza virus in nasal secretions of human beings. For this purpose we used the haemagglutination inhibition test, after neutralizing the non-specific inhibition of the nasal secretions against influenza virus by means of a potent crude filtrate of *Vibrio cholerae.*

METHODS AND MATERIALS.

Influenza vaccines. Monovalent concentrated influenza virus vaccines were kindly placed at our disposal by N. V. Philips Roxane, Weesp (Holland). The vaccines were prepared by ultracentrifugation of infected allantoic fluid. All vaccinations (20 in total) were done with 0.3 ml intracutaneously in the skin of the back. A specimen of blood was taken both at the moment of vaccination and 12 days afterwards.

Collecting the nasal secretions. This took place on the day of vaccination and 12 days subsequently. A small swab of sterile fat-free cotton-wool was inserted into the inferior nasal meatus, and removed 2 hours afterwards. The secretions were pressed out with sterile precautions. They were then examined microscopically for traces of blood. At the same time the benzidine test was done. Haemorrhagic secretions were discarded. Eleven out of the 40 secretions were not entirely sterile.

Crude filtrate of *V. cholerae.* Filtrates with a pH of 7.6 were prepared with the strain 4 Z (kindly sent to us by Professor Dr F. M. BURNET) according to BURNET and STONE (2). As the titre of the non-specific inhibition of nasal secretions may be very high (also, for instance, against the strain PR8), it is necessary to prepare a very potent filtrate. An acceptable filtrate should be capable of eliminating the non-specific inhibition in ferret serum against the strain A-prime Barratt (1949, Eng.) in a dilution of 2 : 3. After the addition of 500 U penicillin and streptomycin per ml, potent crude filtrate was mixed in the proportions of 2 : 1 and 5 : 1 with nasal secretion (drops), and after 48 hours' interaction at 37°C. it was heated for one hour at 56°C. The bloodserum was treated with filtrate in the same way, but only in a proportion of 5 : 1, the interaction lasting from 18 to 24 hours.

The haemagglutination inhibition test. This test was performed with the aid of a micro-method according to VAN DER VEEN and MULDER (8) in tiles of porcelain at a temperature of +2°C. The dilutions were made in drops. In 14 out of the 20 cases the dilution fluid was a 3.8% solution of sodium citrate ($C_3H_4OH\ (COONa)_3 .\ 5\ H_2O$), in order to eliminate any residue of non-specific inhibition by neutralizing free calcium ions (VAN DER VEEN and MULDER (8)). The number of haemagglutination units employed was determined for each experiment separately, being between 2 and 4 A.U. The titres obtained were theoretically corrected for 3 A.U. The nasal secretions of patients, of whom it was known that the titre of the circulating antibodies against the strain used in the vaccine was lower than 12, were used as a control in 6 experiments. Each pair of nasal secretions was titrated against the strain of influenza virus incorporated in the vaccine. In 5 experiments the heterologous strain B-Bon (1943, Austr.) served as a control. Moreover each nasal secretion was examined for agglutinins against chicken red cells.

Mouse protection test. This test was done in one case as a control of the haemagglutination inhibition test. It was performed by mixing dilutions of the nasal secretion with virulent lung suspension containing 10 M.L.D. of the strain A-PR8. The inoculations were done intranasally with 0.05 ml of the mixture. The experiment was concluded after 7 days and autopsy of the surviving mice was performed.

Results.

Table 1 shows the results of the haemagglutination inhibition tests with nasal secretions. Table 2 shows the titres during a period of 51 days in the same patient. Table 3 shows a control of the haemagglutination inhibition test with a mouse protection test. From table 1 it follows that after vaccination the mean titre of the antibody in nasal secretions amounts to 3 °/₀ of that of the circulating antibodies. There is no strict correlation between the figures of the titres of the nasal secretions and those of the circulating antibodies. The nasal secretions of patient Ov were purulent as a consequence of chronic rhinitis, but they did not contain blood. In this case the antititre of the secretions increased after vaccination to a very high value. This may be caused by transsudation of blood serum into inflamed areas of the nasal mucosa besides a very high response of the circulating antibodies. Four of the 20 secretions did not show antibodies after vaccination in a dilution of 12 or more.

Discussion.

The above experiments show that after vaccination it is possible to demonstrate the appearance of antibodies against influenza virus in nasal secretions with the aid of the haemagglutination inhibition test. This simple method would make possible large scale investigations with regard to the problem of respiratory antibodies against influenza virus in human beings. Instances would be: 1. Their appearance after intranasal vaccination with influenza virus, with and without previous pararespiratory vaccination (Fazekaz de St Groth and Donelly (3)); 2. the effect of the very

Tabl

Titres of antibodies in pairs of nasal secretions and corresponding bloodser

Patient	Vaccine	Dilution fluid in the haemagglutination inhibition test	Titres of pairs of nasal secretions used in the	
			Untreated	Chol.filtr. (2 : 1)
Fo	A-PR8	citrate	384/271	<12/<12
Control	. . .	citrate	4344	<12
Ko	A-PR8	citrate	1536/3072	<12/96
Control	. . .	citrate	6144	<12
Br	A-PR8	saline	896/896	<12/112
Control	. . .	saline	1344	<12
Ov	A-PR8	saline	3072/16384	144/9261
Control	. . .	saline	768	<12
Ku	A-PR8	citrate	384/768	18/108
He	A-PR8	citrate	1536/1536	<12/576
Control	. . .	citrate	6144	<12
Wa	A-PR8	citrate	317/896	<12/28
Hey	A-PR8	citrate	317/896	<12/70
Le	A-PR8	citrate	448/896	<12/282
Control	. . .	citrate	1267	<12
Be	A-PR8	saline	768/768	24/24
Dij	A-PR8	saline	896/1344	24/112
Vo	A-PR8	saline	1614/800	34/112
Bo	A-PR8	saline	112/2688	<12/1152
Ga	A′-FM1	citrate	18/18	<12/<12
Schi	A′-FM1	citrate	44/158	<12/20
Zel	A′-FM1	citrate	28/112	<12/20
Rijn	A′-FM1	citrate	19/158	<12/158
Kar	A′-FM1	citrate	79/564	<12/356
Ber	B-Bon (1943, Austr.)	citrate	68/96	17/34
Wolf	B-Bon (1943, Austr.)	citrate	48/634	<12/317

1.

before and after intracutaneous vaccination with strains of influenza virus

against strain vaccine	Titres of pairs of nasal secretions against strain B-Bon (1943, Austr.)	Titres of blood serum against strain used in the vaccine	Ratio antititre blood serum: nasal secretions after vaccination
Chol.Filtr. (5 : 1)	Chol.filtr. (5 : 1)	Chol.filtr. (5 : 1)	
<12/<12	. . .	56/896	. . .
<12		<12	. . .
<12/80	. . .	23/373	5
<12	. . .	<12	. . .
<12/168	<12/<12	42/896	5
<12	<12	<12	. . .
96/9216	<12/<12	168/114688	12
<12	<12	<12	. . .
18/80	. . .	1344/2688	33
<12/384	. . .	218/24576	62
<12	. . .	<12	. . .
<12/22	. . .	93/2688	122
<12/56	. . .	21/746	13
<12/282	. . .	<12/28672	101
<12	. . .	<12	. . .
<12/<12	<12/<12	<12/168	. . .
<12/112	<12/<12	112/2986	26
<12/112	. . .	198/4778	41
<12/768	<12/<12	<12/28672	37
<12/<12	. . .	126/672	. . .
<12/<12	. . .	21/1194	. . .
<12/18	. . .	42/373	20
<12/158	. . .	42/1792	11
<12/356	. . .	21/76458	21
17/34		336/672	20
<12/317		<12/10752	34

Table 2.

Course of the antititre of nasal secretions and bloodserum after intracutaneous vaccination with the strain A-PR8.

Patient	Days after vaccination	Titre of nasal secretion treated with Chol. filtrate (5:1)	Titre of bloodserum	Exp.
Bo	0	<12	<12	1
	14	768	28672	
	14	...	11946	2
	24	768	10752	
	38	121	5376	
	51	136	3548	

high titres of circulating antibodies obtained with adjuvant vaccines (Salk *et al.* (7)) on the titre of nasal secretions; 3. investigations into the antibody titre of secretions from trachea and bronchi (tracheal washings and sputum). We may further say that the results of the above experiments again lend support to the view that vaccination against influenza probably rests on a sound theoretical basis.

Summary.

With the aid of the haemagglutination inhibition test in nasal secretions of human beings it is possible to demonstrate an increase of antibodies against influenza virus after pararespiratory vaccination, provided the non-specific inhibition of the secretions is eliminated by means of enzymes of *Vibrio cholerae*. The antititre of the nasal secretions fluctuated greatly. After intracutaneous vaccination of 0.3 ml in the skin of the back with concentrated vaccine it amounted on an average to 3% of that of the blood titre. The possible importance of this simplified technique for further investigations is pointed out.

References.

1. F. M. Burnet, D. Lush and A. V. Jackson, Brit. J. Exp. Path. **20**, 377, 1939. – 2. F. M. Burnet and J. D. Stone, Austr. J. Exp. Biol. Med. Sc. **25**, 227, 1947. – 3. S. Fazekaz de St Groth and M. Donelly, Austr. J. Exp. Biol. Med. Sc **28**, 15, 1950. – 4. Th. Francis, Sciene **91**, 198, 1940. –

Table 3.

Mouse protection test with a pair of nasal secretions collected before and after intracutaneous vaccination with the strain A-PR8 and treated with cholera filtrate (2 : 1) [1]).
The haemagglutination inhibition titre of the two secretions was <12 and 282 (Patient Le of table 1).

Pair of secretions Chol.filtr. (2 : 1)	Dilution of secretions						Control 10 × M.L.D.
	6	12	24	48	96	192	
Le I	++++(5) ++++(6) ++++(6) ++++(6)	++++(5) ++++(5) ++++(6) ++++(7)	...	...	...	...	++++(4) ++++(5) ++++(5) ++++(6)
Le II	0 0 0 0	0 0 + ++	0 + + ++	++ +++ +++ +++	++ +++ ++++(6) ++++(7)	+ +++ ++++(6) ++++(7)	

---

[1]) 0 — ++++ = degree of lung consolidation at death or at autopsy on the 7th day.
The figure between brackets indicates the day after inoculation of spontaneous death from influenzal pneumonia

5. Th. Francis and I. J. Brightman, Proc. Soc. Exp. Biol. Med. **48**, 116, 1941. – 6. Th. Francis, H. E. Pearson, E. R. Sullivan and Ph. M. Brown, Am. J. Hyg. **37**, 294, 1943. – 7. J. Salk, A. M. Laurent and M. Lynch Bailey, Am. J. publ. Hlth. **41**, 669, 1951. – 8. J. van der Veen and J. Mulder, Studies on the antigenic composition of human influenza virus A strains. Onderz. en meded. Instit. Praev. Geneesk. **6**, 1950, Leiden (p. 7 and 18).

(From the Virus Laboratory of the Clinic of Internal Medicine,
University of Leyden).

STUDIES ON THE ANTIGENIC BEHAVIOUR OF EGG- AND EGG-MOUSE-EGG-LINES OF STRAINS OF INFLUENZA VIRUS, WITH THE AID OF THE HAEMAGGLUTINATION-INHIBITION TEST ¹)

by

J. MULDER and L. M. BRANS

(Received November 18, 1951).

INTRODUCTION.

HIRST (1) was the first to demonstrate that pure egg-lines of strains of influenza virus A in the cross haemagglutination inhibition test show a serological pattern that differs from the ferret-mouse lines. A similar observation was also mentioned by SUGG (5), who compared egg-lines and egg-mouse-egg-lines of the strain Cam (1946, Austr.).

When comparing the antigenic patterns of strains of influenza virus, little attention has so far been paid to the passage formula of the strains. Many workers, for example, use the strain FM1 (1947, U.S.A.) as a reference strain for the A-prime group, without mentioning whether it underwent any mouse passages. This strain was sent us by courtesy of Dr A. Cox. He informed us that the strain, originally isolated in the chick embryo (RASMUSSEN *et al.* (7)), had undergone 8 consecutive mouse-passages. Strains of influenza virus B, which since 1940 have been almost exclusively isolated in chick embryo, are in most cases compared with the Lee strain, which underwent many ferret and mouse passages. Hence

---

¹) We are indebted to Dr G. K. HIRST (New York) and Dr J. Y. SUGG (New York) for sending us the strains of influenza virus Ala and Cam. Financial support was provided by the Institute for Preventive Medicine, Leyden; N. V. Philips Roxane, Weesp; the State Department of Science; the Jan Dekker Fund, and the Curaçao Fund for Preventive Medicine.

it seemed important to us to investigate how egg-lines and egg-mouse-lines of the same influenza strain behave mutually, and towards strains of homologous and heterologous sub-groups. Use was made of the haemagglutination inhibition test and ferret sera. The non-specific inhibition was removed from the antisera, because in certain circumstances the latter may considerably affect the level of the antititres (VAN DER VEEN and MULDER (7, p.24)).

As most of our experiments were done with strains of the A-prime group, which at present are almost always isolated in the chick embryo, we have chiefly compared pure egg-lines with egg-mouse-egg-lines.

MATERIALS AND METHODS.

Antigens. For the haemagglutination inhibition test we used a pool of infected allantoic fluid from embryonated eggs that were incubated for 2 days. After centrifuging for 10 minutes at 3500 revolutions the fluid was diluted with 3 parts physiological saline, to which merthiolate was added (final dilution 1 : 10.000). The pools were stored in bottles of alkaline-free glass at 2°C.

Antisera. Antisera were prepared from ferrets that were anaesthetised with ether and intranasally infected with 1 ml of infected allantoic fluid. For each strain one ferret was used. After 12 days the animals were bled. In a control test each ferret serum was tested against the strains B-Bon (1943, Austr.), A-swine 15 (1930, U.S.A.), A-WS (1933, Eng.), A (1941, Ned.) and A-prime-FM1 (1947, U.S.A.), which are regularly used in our laboratory. At the same time it was ascertained whether the pre-infection ferret serum contained antibodies against one of these strains in a minimal dilution of 12. All the ferret sera were treated with a crude filtrate of *V. cholerae* to eliminate the non-specific inhibition (MULDER and VAN DER VEEN (2)). The antisera were stored at 2° C. without a preservative.

Cross haemagglutination inhibition tests. These were performed at 2° C. with the aid of a micro-method (VAN DER VEEN and MULDER (7, p. 7)). The number of haemagglutination units of the virus was determined for each test separately (mostly between 2 and 4 A.U.), the antititres being theoretically corrected for 3 A.U. A normal ferret serum, known to contain a high amount of non-specific inhibitor, was used in each experiment as a control for the absence of inhibitor after treatment with the same crude filtrate of *V.cholerae* which was used for the antisera. Moreover, each filtrate was examined beforehand for its potency against strains known to be highly susceptible to non-specific inhibition [strain Barratt (1947, Eng.) (egg-line) and A (1941, Ned.) (ferret-mouse-egg-line)].

Egg protection test. In this test 4 eggs were used per serum dilution. The M.E.I.D.$_{50}$ of the allantoic fluid was estimated twice (before and on the day of the protection test) so that the actual number of M.E.I.D.$_{50}$

of the virus used in the test could be calculated. The titres obtained in the protection test were corrected according to Reed and Muench (4).

Mouse adaptation. The strains were adapted to the mouse lung by means of intranasal inoculation. The strain Cam was kindly sent to us as an egg-line and an egg-mouse-line by Dr J. Y. Sugg, New York. The virulence of the mouse strains was not determined separately. After the appearance of the first fatal virus pneumonias the mouse passages were continued with 5 to 7 passages. All the strains were fatal to mice within 10 days, when inoculated with 0.05 ml of a 10% suspension of infected mouse lung. For the preparation of the antiserum against the mouse-lines infected allantoic fluid was used, with the exception of the strain Ala (1941, U.S.A.), of which an infected mouse lung suspension was used. The number of egg-passages after mouse adaptation was different for the various strains.

In the following text the egg-line of an influenza virus strain is indicated by E, the egg-mouse-egg-line by EME. The same holds good for the antiserum belonging to it. The exact passage formula will be found in the tables. E_x denotes that the number of egg passages is unknown to us.

Experiments.

Crossings with E- and EME-lines of strains from the A-prime group. Incidentally performed crossings between egg- and egg-mouse-egg-lines of strains of influenza virus were published on a previous occasion (van der Veen and Mulder (7, p.36)). They related to only 4 pairs of experiments with strains from the A-prime group [Barratt (1947, Eng.), Gg (1947, Sweden) and Heer (1949, Ned.)]. The experiments (Table 1)

Table 1.

Cross haemagglutination inhibition tests with egg (E)- and egg-mouse-egg (EME)-lines of influenza virus strains belonging to the subgroup A-prime [1].

ANTISERUM		STRAINS	
		E	EME
HOMOLOGOUS	E	1	1.5
	EME	1	8
HETEROLOGOUS	E	1	2
	EME	1	4.1

[1] The titres obtained in the experiments are added and expressed in the ratio ΣE : ΣEME.

Table 2.

Cross haemagglutination inhibition tests with egg- and egg-mouse-egg-lines of four strains of influenza virus belonging to the subgroup A-prime (four experiments).

FERRET ANTISERA	STRAINS								Exp.
	E	EME	E	EME	E	EME	E	EME	
	Cam (1946, Austr.) E_{94}	Cam (1946, Austr.) $E_{38}M_{60}E_2$	Barratt (1947, Eng.) E_xE_{52}	Barratt (1947, Eng.) $E_xE_1M_{20}E_6$	Heer (1949, Ned.) E8	Heer (1949, Ned.) $E_8M_{20}E_{10}M_7E_{20}$	Baak (1951, Ned.) E_{11}	Baak (1951, Ned.) $E_7M_{17}E_2$	
Cam (1946, Austr.) E_{92} E	**1422**	1219	44	152	158	610	<12	70	1
Cam (1946, Austr.) $E_{38}M_{60}E_1$ EME	79	**17378**	564	1219	633	4344	81	1267	
Barratt (1947, Eng.) E_xE_{21} E	14	242	**3548**	1219	1219	2172	305	896	2
Barratt (1947, Eng.) $E_xE_1M_{20}E_3$ EME	79	1086	2845	**7741**	1935	2438	305	1267	
Heer (1949, Ned.) E_{11} E	141	768	5068	4344	**2845**	8689	448	2534	3
Heer (1949, Ned.) $E_8M_{20}E_7$ EME	448	768	711	1267	633	**17378**	44	2258	
Baak (1951, Ned.) E_6 E	<12	<12	2534	610	152	242	**1267**	1267	4
Baak (1951, Ned.) $E_7M_{17}E_1$ EME	141	356	564	484	152	4344	896	**9031**	

give only approximate results, since we did not perform complete crossings between the 3 strains. Our provisional conclusion from them was that the mouse-adapted egg-lines of A-prime strains are, generally speaking, better antigens in the haemagglutination inhibition test than the egg-lines. The greatest differences in the titres were observed when sera EME were crossed with the homologous E and EME strains, the titres of the E strains being in most cases very low (ratio Σ E: Σ EME = 1 : 8).

Table 2 shows the crossings with 4 A-prime strains. They were performed in the course of 4 experiments on 4 consecutive days with the same antigens and the same chicken red cells.

Table 3 shows the titres found and added, in the ratio Σ E : ΣEME, which resemble those of table 1. The only experiments where the EME-line is obviously less influenced than the homologous E-line, are those with the anti E-serum of the strains Barratt (1947, Eng.) and Baak (1951, Ned.) against both lines of the strain Barratt. The greatest difference in titres was again observed when the serum EME was crossed with the homologous E- and EME-lines (ratio ΣE : ΣEME = 1 : 11.6).

Table 3.

Cross haemagglutination inhibition tests with egg (E)- and egg-mouse-egg (EME)-lines of 4 influenza virus strains belonging to the subgroup A-prime [1]).

ANTISERUM		STRAINS	
		E	EME
HOMOLOGOUS	E	1	1.4
	EME	1	11.6
HETEROLOGOUS	E	1	1.3
	EME	1	3.7

[1]) The titres obtained in the experiments are added and expressed in the ratio ΣE : ΣEME.

Crossings of E-lines and EME-lines of strains of the A-(PR8) group. Table 4 shows the crossings of the E-lines and the EME-lines of the strains Ala (1941, U.S.A.) and

Table 4.

Cross haemagglutination inhibition tests with egg- and egg-mouse-egg-lines of two strains of influenza virus belonging to the A-(PR8)-group (one experiment).

FERRET ANTISERA	STRAINS			
	E	EME	E	EME
	Ala (1941, U.S.A.) E_{10}	Ala (1941, U.S.A.) E_6M_{18} E_3	965 T (1943, U.S.A.) E_xE_4	965 T (1943, U.S.A.) $E_xE_4M_{16}E_1$
Ala (1941, U.S.A.) E_7 E	**9031**	34755	8689	3870
Ala (1941, U.S.A.) E_6M_{18} EM	2845	**30963**	4344	2438
965 T (1943, U.S.A.) E_xE_5 E	2534	9753	**6144**	1219
965 T (1943, U.S.A.) $E_xE_4M_{16}E_1$ EME	11379	49152	17378	**9753**

Table 5.

Cross-egg protection and corresponding haemagglutination inhibition tests with the antisera of the E- and EME-line of the strain A-Ala (1941, U.S.A.).

FERRET ANTISERA	STRAINS			
	Egg-protection test		Haem. aggl. inh. test	
	E	EME	E	EME
	Ala (1941, U.S.A.) E_{10}	Ala (1941, U.S.A.) $E_6M_{18}E_3$	Ala (1941, U.S.A.) E_{10}	Ala (1941, U.S.A.) $E_6M_{18}E_3$
	30 × M.E.I.D.$_{50}$	70 × M.E.I.D.$_{50}$	3 A.U.	3 A.U.
Ala (1941, U.S.A.) E_7 E	**96**	2173	**6451**	78024
Ala (1941, U.S.A.) E_6M_{18} EME	30	**2173**	2560	**69512**

965 T (1943, U.S.A.) 1)). The E- and the EME-line of the strain Ala behave as those of the strains from the A-prime group. A pecularity of the strain 965 T, however, is that the EME-line is a less good antigen than the homologous E-line.

Cross egg-protection tests with the E- and EM-line of strain Ala (1941, U.S.A.). Table 5 shows the titres obtained with a cross-egg-protection test with the E- and EME-line of the strain Ala (1941, U.S.A.). Though the infective doses used proved to be slightly different for both lines of virus, the titres obtained have about the same pattern as those in the corresponding haemagglutination inhibition test, showing a very low value for the egg-line against the serum EM. Two control tests confirmed this result.

Crossings with ferret-egg- and ferret-egg-mouse-egg-strains from the A-PR8 group. Table 6 shows an instance of such an experiment. It is seen that the ferret-egg-line of the strain A-Sch (1941, Ned.) behaves in the same

Table 6.

Cross haemagglutination inhibition tests with ferret-mouse-egg-lines and a ferret-egg-line of strains of influenza virus belonging to the A-PR8-subgroup (one experiment) *).

FERRET ANTISERA	STRAINS			
	FME	FME	FE	FEME
	A (1941, Ned.) $F_3M_{38}E_{37}$	A-Sl (1941, Ned.) $F_4M_{36}E_{27}$	A-Sch (1941, Ned.) F_3E_{10}	A-Sch (1941, Ned.) $F_3E_6M_{12}E_5$
A (1941, Ned.) $F_3M_{38}E_{31}$ FME	**6140**	4780	120	4780
A-Sl (1941, Ned.) $F_4M_{36}E_{25}$ FME	12290	**19110**	80	11950
A-Sch (1941, Ned.) F_3E_8 FE	3410	5970	**3410**	28670
A-Sch (1941, Ned.) $F_3E_6M_{12}E_5$ FEME	4100	2690	1380	**14340**

*) Titres corrected in decimals.

---

1) Isolated by Dr R. M. Taylor.

manner as the egg-lines of most strains from the A-prime group from table 2 and 3.

Crossings of E- and EME-lines with strains of heterologous sub-groups. Tables 7a and 7b, 8a and 8b show these crossings with the E- and the EME-line of the strains Ala (1941, U.S.A.), and Cam (1946, Austr.). From these experiments it is seen that, with an occasional exception (WS × Cam (E-line)), the EME-lines are slightly more influenced by antisera of strains from a heterologous sub-group than their E-lines, but the overall serological pattern of EME-lines proves to be such that they clearly belong to the same sub-group as the E-lines. The serum Cam-EME shows a serological polyvalence with respect to the A-PR8 group, which was also noticed by Taylor (6) and van der Veen and Mulder (7, p. 51). The serum Cam-E does not show this overlapping with the heterologous A-PR8 group in a marked degree. An earlier experiment with another

Table 7a and 7b.

Cross haemagglutination inhibition tests with the egg- and egg-mouse-egg-line of the strain A-Ala (1941, U.S.A.) and heterologous strains (table 7a and 7b are from one experiment).

Table 7a.

FERRET ANTISERA	STRAINS		
	E	EME	
	Ala (1941, U.S.A.) E_8	Ala (1941, U.S.A.) $E_6M_{18}E_2$	Homologous strain
Swine-15 (1930, U.S.A.) $F_xM_xE_{37}$ FME	<12	<12	**1152**
WS (1933, Eng.) $F_xM_xM_{207}E_{35}$ FME	<12	144	**4608**
PR8 (1934, U.S.A.) $F_{198}M_{593}E_{55}M_3E_1$ FME	144	4096	**40960**
A (1941, Ned.) $F_3M_{38}E_{31}$ FME	80	4608	**4096**
FM1 (1947, U.S.A.) $E_xM_8E_{24}$ EME	12	108	**5376**

Table 7b (belongs to table 7a, p. 146).

FERRET ANTISERA	STRAINS						
	E	EME	FME	FME	FME	FME	EME
	Ala (1941, U.S.A.) E_8	Ala (1941, U.S.A.) $E_6M_{18}E_2$	Swine-15 (1930, U.S.A.) $F_xM_xE_{53}$	WS (1933, Eng.) $F_xM_xM_{207}E_{50}$	PR8 (1934, U.S.A.) $F_{198}M_{593}E_{55}M_3E_2$	A (1941, Ned.) $F_3M_{38}E_{40}$	FM1 (1947,U.S.A.) $E_xM_8E_{30}$
Ala (1941, U.S.A.) E_7 E	**8192**	36384	<12	80	2560	9216	75
Ala (1941, U.S.A.) E_6M_{18} EM	2304	**40960**	<12	96	10240	6144	75

Table 8b (belongs to table 8a, p. 148).

FERRET ANTISERA	STRAINS						
	E	EME	FME	FME	FME	EME	EME
	Cam (1946, Austr.) E_{93}	Cam (1946, Austr.) $E_{38}M_{60}E_2$	WS (1933, Eng.) $F_xM_xM_{207}E_{44}$	PR8 (1934, U.S.A.) $F_xM_xM_{109}E_2$	A (1941, Ned.) $F_3M_{38}E_{45}$	FM1 (1947,U.S.A.) $E_xM_8E_{24}$	Heer (1949, Ned.) $E_8M_{20}E_{12}$
Cam (1946, Austr.) E_{92} E	**2560**	1536	14	21	84	288	1365
Cam (1946, Austr.) $E_{38}M_{60}E_1$ EME	160	**24576**	<12	1610	3548	1152	6828

Table 8a and 8b.

Cross haemagglutination inhibition tests with the egg- and egg-mouse-egg-line of the strain A-prime-Cam (1946, Austr.) and heterologous strains (table 8a and 8b are from two experiments).

Table 8a.

FERRET ANTISERA	STRAINS	
	E	EME
	Cam (1946, Austr.) E_{92}	Cam (1946, Austr.) $E_{38}M_{60}E_{1}$
Swine-15 (1930, U.S.A.) $F_xM_xE_{37}$ FME	<12	<12
WS (1933, Eng.) $F_xM_xM_{207}E_{35}$ FME	14	<12
PR8 (1934, U.S.A.) $F_{198}M_{593}E_{82}$ FME	<12	<12
A (1941, Ned.) $F_3M_{38}E_{31}$ FME	12	28
FM1 (1947, U.S.A.) $E_xM_8E_{24}$ EME	198	1792
Heer (1949, Ned.) E_{23} E	168	1344

antiserum (Cam E_{69}) showed that the egg-strain already possesses this polyvalent potency. This proves that the serological pattern of one ferret serum may be liable to rather considerable variations. This follows also from the differences in titres that were found by us in the crossings with the E- and the EME-line of the strain Cam, when compared with the results of Sugg with the same passage formula of these strains (Sugg (5)).

It is remarkable that the titres of the E-line Ala against antisera of the homologous sub-group are almost equal to those of the Ala-EME-line against the heterologous subgroup-antiserum FM1 (Table 7a). The titre of the E-line Cam against the homologous antiserum EME is also much lower than the titre of the strains PR8 and A (1941, Ned.) against the EME-antiserum of the Cam- strain (table 8b).

Crossings of the E-line and the EME-line of

Table 9a and 9b.

Cross haemagglutination inhibition tests with the egg- and egg-mouse-egg-line of the strain B-Bon (1943, Austr.) and other B-strains (Tables 9a and 9b are from two experiments).

Table 9a.

FERRET ANTISERA	STRAINS		
	E	EME	Homologous strain
	Bon (1943, Austr.) E_{55}	Bon (1943, Austr.) $E_{57}M_{26}E_5$	
Lee (1940, U.S.A.) $F_8M_{137}E_{172}$ FME	81	242	**2845**
Bon (1943, Austr.) E_{51} E	2048	6144	**2048**
Paddington (1943, Eng.) $F_xE_xE_3$ FE	203	271	**11585**
Crawley (1946, Eng.) E_{14} E	2896	4344	**2258**
Warner (1948, Austr.) E_xE_7 E	512	1219	**711**
Bud. 1/49 (1949, Hung.) E_xE_5 E	1448	4344	**1267**
Todd (1950, Eng.) E_{10} E	724	2172	**1742**

the B-strain Bon (1943, Austr.). They will be found in table 9a and 9b. This strain too shows the phenomenon that the EME-line is a better antigen in the cross haemagglutination inhibition tests than the E-line. The differences, however, are not very marked.

The strain FM1. The strain that is used in our laboratory has a passage-formula E_xM_8E. E_x is unknown to us. It may, therefore, be possible that the antiserum of this strain behaves as an EME-antiserum. In order to obtain confirmation the strain was passed once more in mice, so that the final passage formula read as follows: $E_xM_8E_{21}M_{13}E$. Cross-tests between the E_xM_8E-line and the $E_xM_8E_{21}M_{13}$ E-line (table 10) showed that they had the same titres, so that it is very probable that the E_xM_8E-line of the

Table 9b (belongs to Table 9a, p. 149).

FERRET ANTISERA	STRAINS							
	E	EME	FME	FE	E	E	E	E
	Bon (1943, Austr.) E_{55}	Bon (1943, Austr.) $E_{57}M_{26}E_5$	Lee (1940, U.S.A.) $F_8M_{137}E_{160}$ $M_{10}E_8$	Padd. (1943, Eng.) $F_xE_xE_3$	Crawley (1946, Eng.) E_{13}	Warner (1948, Austr.) E_xE_7	Bud 1/49 (1949. Hung.) E_xE_5	Todd (1950, Eng.) E_{11}
Bon (1943, Austr.) E_{51} E	**1625**	4876	362	406	1129	356	633	633
Bon (1943, Austr.) $E_{57}M_{26}E_3$ EME	2580	**8689**	813	645	711	711	633	711

Table 10.

Cross haemagglutination inhibition test with the egg-mouse-egg- and egg-mouse-egg-mouse-egg-line of the strain A-prime FM1 (1947, U.S.A.) (one experiment).

FERRET ANTISERA	STRAINS	
	EME	EMEME
	FM1 (1947, U.S.A.) $E_xM_8E_4$	FM1 (1947, U.S.A.) $E_xM_8E_{21}M_{13}E_{15}$
FM1 (1947, U.S.A.) $E_xM_8E_{46}$ EME	**12288**	18062
FM1 (1947, U.S.A.) $E_xM_8E_{21}M_{13}E_6$ EMEME	9753	**14336**

strain FM1 behaves as a mouse-line. This is important when cross experiments of this strain are performed with the haemagglutination inhibition test, since the titre of recently isolated A-prime strains may be low with the EME-line of FM1, which, however, does not imply less relationship to this strain (VAN DER VEEN and MULDER (7, p. 47).

DISCUSSION.

We are not able to give an explanation of the above mentioned serological patterns in crossings of E-lines and EME-lines. SUGG (5) suggests the possibility that egg-strains are antigenically not homogeneous, and that mouse-adaptation may give rise to a selection deviating antigenically from the egg-line. In routine examination of the antigenic composition of influenza strains however the data obtained are significant, since they show it to be very important that the passage formula of the strains used should be mentioned. The possibility exists that the antititres of the EME-antisera against the homologous and the heterologous egg-lines of strains belonging to the same subgroup are low, and occasionally very low.

Further investigation must show whether this also holds good for the B-group.

Summary.

Egg- and egg-mouse-egg-lines of strains of influenza virus were compared with the aid of the haemagglutination inhibition test.

In most experiments it was found that the crossing of an antiserum of an egg-mouse-egg-line against the homologous egg-line or against a heterologous egg-line of a strain belonging to the same subgroup yields a low or very low antititre.

The experiments were performed with the technical assistance of Miss I. de Nooijer.

References.

1. G. K. Hirst, J. Exp. Med. **78**, 407, 1943. – 2. J. Mulder and J. van der Veen, Acta med. brev. Neerl. **16**, 57, 1948. – 3. A. F. Rasmussen, J. C. Stokes and J. E. Smadel, Am. J. Hyg. **47**, 142, 1948. – 4. L. J. Reed and H. Muench, Am. J. Hyg. **27**, 493, 1938. – 5. J. Y. Sugg, J. Bact. **58**, 399, 1949. – 6. R. M. Taylor, Am. J. Publ. Health **39**, 171, 1949. – 7. J. van der Veen and J. Mulder, Studies on the antigenic composition of human influenza virus A strains. Onderz. en meded. Inst. Praev. Geneesk. **6**, 1950, Leiden.

(Forest Research Station T.N.O., Wageningen, Netherlands).

A PRELIMINARY STUDY ON THE CULTURE OF *DISCOMYCETES*, ESPECIALLY THE PERFECT STAGE

by

J. GREMMEN

(Received December 4, 1951).

LITERATURE.

In 1891 BREFELD (2) was the first to report on the cultivation of *Discomycetes* in vitro. A large number of species was investigated by him. He described a number of very interesting cases of formation of conidia up till then unknown. His experiments showed that apothecia in culture were rarely obtained. Only in a single species *viz.*, *Pyrenopeziza tamaricis* (Roum.) one mature apothecium was obtained in a seven months old culture.

In 1931 GREGOR WILSON (9) mentioned the development of mature apothecia in cultures of *Dermatea livida* (B. et Br.) Phill. Although a number of strains of this fungus were able to form this stage, many of them failed to do so.

The function of the microconidia (spermatia) was found by DRAYTON (4) in 1932. These very small cells were already well-known since long, but their function up till then had been unexplained. In his cultures always some definite organs were obtained which he designed as "receptive bodies". By spermatization of these organs with microconidia from other isolates he succeeded in obtaining fully mature apothecia. By application of this technique DRAYTON (5, 6) succeeded in obtaining mature apothecia of the following fungi: *Sclerotinia gladioli* Drayton, *Sclerotinia convoluta* Drayton, and from fungi belonging to the complex *Botrytis cinerea* Pers. (GROVES and DRAYTON (11)).

In 1938 BUDDIN found a *Botrytis* species on tubers of *Gladiolus*. In a culture of this fungus small sclerotia abunded. By means of the technique introduced by DRAYTON (4,5) apothecia were cultivated. This stage belonging to the genus *Sclerotinia* was afterwards described by DENNIS and WAKEFIELD (3) and named *Sclerotinia Draytoni* Budd. and Wakef.

In 1939 WOLLENWEBER (21) gave a detailed account of his work on *Pezicula*. He was able to obtain the perfect stage in some species.

WHITE (20) in his thesis on the genus *Rutstroemia* reports that *R. longipes* (Cooke and Peck) White easily developed apothecia on nutrient agar.

In 1944 WHETZEL (19) described a new genus of the *Sclerotiniaceae* which he named *Coprotinia*. This monotypic genus with the species *C. minutula* nov. spec. produced apothecia in vitro.

NOBLE (14) in 1948 in a fungus isolated from clover seed could obtain the perfect stage which she named *Sclerotinia spermophila* nov. spec.

In 1950 WATERMAN and CASH (17) published a report on a leaf disease of poplars caused by a fungus, which when cultivated developed small sclerotia. Using DRAYTONS technique they succeeded in obtaining mature apothecia of this fungus, which was named *Septotinia populiperda* nov. spec.

When the literature on the culture of the perfect stage of *Discomycetes* is perused results appear to be very meagre, and the same keeps true for other groups of fungi. Also in the *Pyrenomycetes* the formation of perithecia is scanty. Only in some definite genera this has proved possible (*Pleospora*, *Chaetomium*, *Glomerella*) and also in a few miscellaneous cases (*Cryptodiaporthe salicina*, *Mycosphaerella*).

The investigations of DRAYTON (4,5) point at sexuality as the main factor for the formation of the perfect stage, although doubtless other important agents will exist. Definite strains of one kind of fungus might form apothecia, while other strains might lack this possibility. In all the above mentioned species of fungi which had formed the perfect stage microconidia were always found. In many other groups, however, these cells have been never detected whilst then never any apothecium developed in culture. Although in some of them a small stipe was found no further development was noted (*Helotium* species). Perhaps fertilization will act here by means of a trichogyne (*Pyronema*). Then the lack of formation of apothecia would be due to outward conditions such as nutrition, light or temperature.

PURPOSE AND METHODS.

The purpose of this study was to cultivate a number of *Discomycetes* and more particularly to investigate whether the development of mature apothecia might be induced. It would be of great importance for the taxonomy of this group of fungi to arrive in growing these fructifications. The taxonomy for instance of the genus *Mollisia* needs a thorough revision. All species of this genus are found on decaying material (stems and leaves), and appear quite variable.

When the apothecia could be grown on a precisely defined medium in the sense of WOLLENWEBER and REINKING (22), we would be able to study them in vitro and learn whether those different forms might belong to a few species possessing a strong variability.

For phytopathological research it would be of great value to cultivate apothecia in the laboratory all over the year so that mature apothecia (i.c. ascospores) for inoculation tests might always be disposed of. In the past years the author therefore collected a large number of species, using many of them in culture experiments.

Cultures were obtained by means of the naturally ejaculated ascospores. In a few cases isolates were grown from carefully desinfected tissue. Thin and transparant petri-dishes were used and after sterilization filled with sterile water agar. The used apothecia for this purpose must contain mature asci and should be controlled in advance as to this. By means of a droplet of a mixture of paraffin-gelatine the apothecia were made to stuck to the cover of the dish so that the hymenium was directed to the agar. For this manipulation the disk was inverted and then turned over again, so that the ascospores when ejaculated were shot down into the agar.

In this way multi-ascosporic isolates were secured. It is possible to examine the germination of these spores under the low power of the microscope in the inverted dish. Contaminations with bacteria or yeasts will soon be detected. Many ascospores initially one-celled soon became two- or more-celled after ejaculation. Instead of using water agar a well-filtered solution of potato-glucose with agar is also suitable. When germination is sufficient the young colonies were transferred to other media e.g. cherry decoction agar, potato-dextrose agar or malt agar. Many isolates were also grown on sterile stems or branches. For a long cultivation in flasks on grains of wheat or rice the introduction of stems or branches sometimes proved suitable. As these stems appeared to be hydrolysed by the autoclaving, sterilization with narcotics (ethyl ether) was tested in a few cases. During at least a week the material was kept in a closely stoppered bottle containing some ether. If sterile and after the ether was evaporated the material was brought into sterile dishes or tubes and inoculated with the fungus. In order to study the influence of light cultures were sometimes placed in diffuse or even in direct sunlight for a moment. It is known from literature that light can induce fructification, although glass absorbs the ultra-violet part of the spectrum which is responsable for this phenomen (Stevens (15)).

In order to examine the influence of temperature, the cultures were alternately kept at various temperatures. In some cases the isolates were grown together with other organisms, for the detection of any stimulating influence on formation of apothecia. For this purpose some yeasts were used. Up till now no stimulating action has been noted, whilst the yeasts were mostly very inconvenient in vitro. Cultures grown together with the fungus *Phycomyces Blakesleeanus* Burgeff showed no particularities.

Results of the culture in vitro.

The following Discomycetous fungi were grown in vitro. Only six of them produced the perfect stage. The other fungi failed to do so. The time of cultivation varied from 2 to 12 months. Collecting and identifying was performed by the author. The nomenclature is in agreement with the international Rules.

Operculates:

Aleuria cerea (Sow. ex Fr.) Boud., on sandy soil; *Barlaea constellatio* (B. et Br.) Rehm, on forest soil; *Ciliaria scutellata* (Fuck.) Rehm, on forest soils; *Desmazierella acicola* Lib., on dead needles of pines; *Leotia lubrica* (Scop.) Pers., on forest soils.

Inoperculates:

Arachnopeziza aurelia (Pers. ex Fr.) Fuck., on acorns; *Belonioscypha campanula* (Nees ex Fr.) Rehm, on culms of grasses; *Calloria fusarioides* (Berk.) Fries, on stems of dead nettle; *Cenangium acicolum* (Fuck.) Rehm, on dead needles of pine; *Cenangium ferruginosum* Fries, on branches of pine; *Colpoma quercinum* (Pers. ex Fr.) Wallr., on branches of oaks; *Crumenula sororia* Karst., on

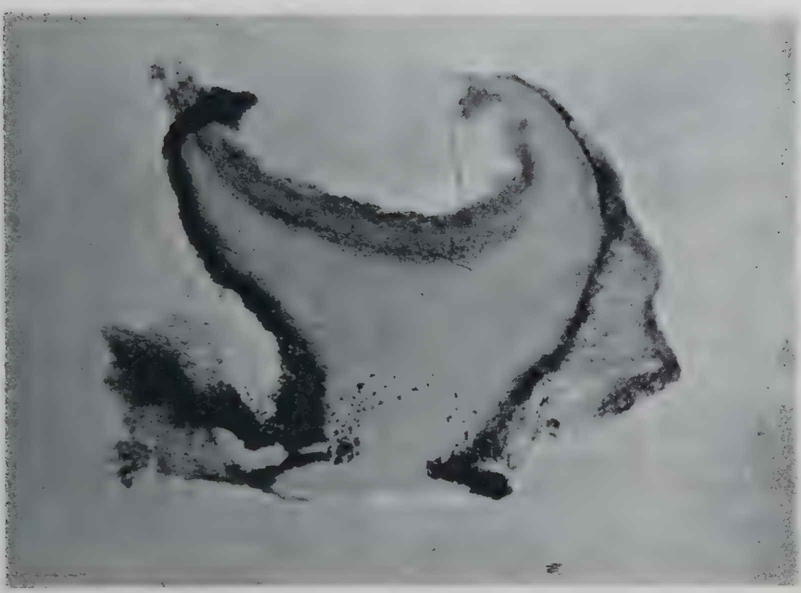


Fig. 1. *Encoelia fascicularis* (Fr.) Karst.
Transverse section through cultivated apothecium.

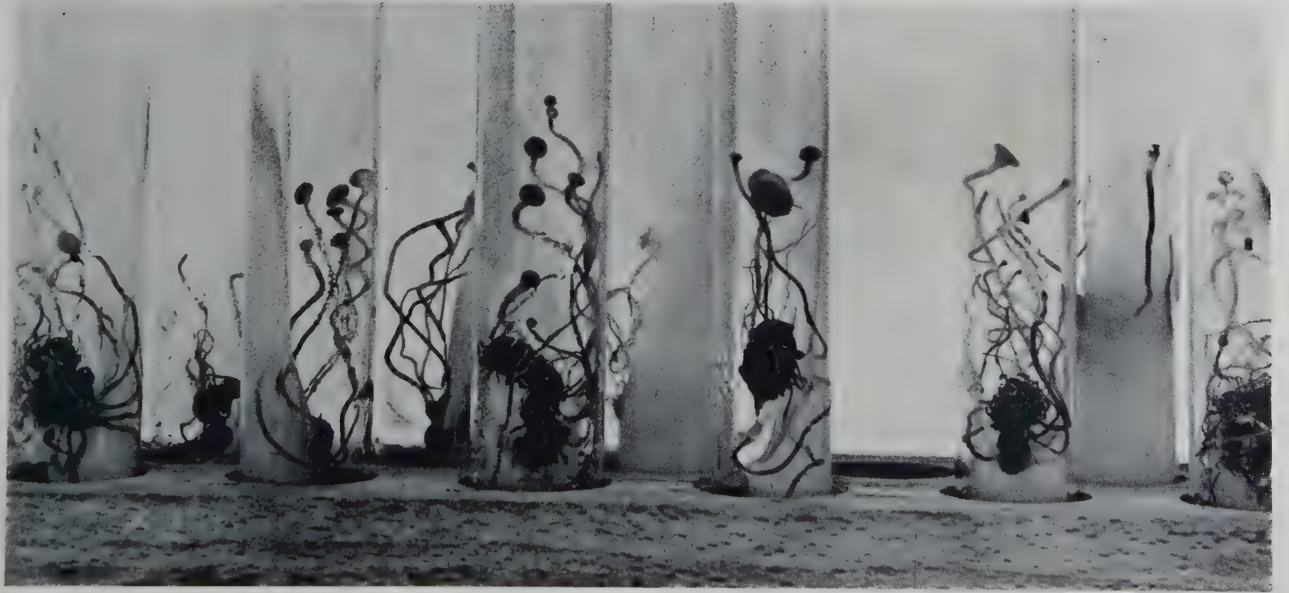


Fig. 2. *Sclerotinia sclerotiorum* (Lib.) de Bary.
Tubes with cultivated sclerotia and apothecia.

branches of pines; *Cyathicula coronata* (Bull.) de Not., on stems of *Artemisia*; *Dasyscypha calycina sensu* Fuck., on branches of larch; *Dasyscypha Willkommii* (Hart.) Rehm, on "canker" of european larch; *Encoelia fascicularis* (Fr.) Karst., on branches of ash and poplar; *Fabraea ranunculi* (Fr.) Karst. on living leaves of *Ranunculus*; *Helotium ciliatosporum* (Fuck.) Boud., on stems of herbaceous plants; *Helotium citrinum* (Hedw.) Fries, on branches of willow; *Helotium fructigenum* (Bull.) Karst., on fruits of oak; *Helotium herbarum* (Pers.) Fries, on stems of nettle; *Helotium scutula* (Pers. ex Fr.) Karst., on dead herbaceous stems; *Helotium sublenticulare* Fries, on decaying wood; *Lachnum acutipilum* Karst., on culms of grasses; *Lachnum sulphureum* (Pers. ex Fr.) Karst., on nettle; *Lachnum virgineum* (Batsch. ex Fr.) Karst., on stems of *Calluna* and branches of oak; *Mollisia cinerea* (Fr.) Karst., on branch of oak; *Mollisia* species, on stems of *Anthriscus*; *Pezicula cinnamomea* (Fr.) Sacc., on twig of *Alnus incana*; *Pezicula livida* (B. et Br.) Rehm, on branches of Douglas fir and larch; *Phialea acuum* (Alb. et Schw. ex Fr.) Rehm, on needles of pine; *Rutstroemia firma* (Pers.) Karst., on branch of oak; *Rutstroemia luteo-virescens* (Rob.) White, on branch of maple; *Rutstroemia Sydowiana* (Rehm) White, on leaves of oaks; *Sclerotinia pseudotuberosa* Rehm, on fruits of oaks; *Sclerotinia sclerotiorum* (Lib.) de Bary, on living plants of beans; *Sclerotinia tuberosa* (Hedw.)Fuck., on rhizomata of *Anemone*; *Trichobelonium obscurum* Rehm, on twigs of *Calluna*; *Trichobelonium retincolum* (Rab.) Rehm, on stems of *Phragmites*; *Trichoscypha subtilissima* (Cooke) Boud., on branches of Douglas fir; *Velutaria rufo-olivacea* (Alb. et Schw. ex Fr.) Fuck., on twigs of *Rubus fruticosus*.

SPECIES PRODUCING THE PERFECT STAGE IN CULTURE.

Cenangium ferruginosum Fr., Vet. Akad. Handl. p. 361, 1818.

The germination of the ascospores as well as further development was very slow. After a fortnight a small colony was observed. On cherry decoction agar the colour of the mycelium was yellowish-green. This is very characteristic for the fungus. After 4 weeks black pycnidia-like bodies were seen oozing a brown till yellowish-brown liquid at maturity. This liquid contained a great number of one-celled, hyaline, bacilliformed cells measuring (3.5—4.5) × (1—1.5)μ. These cells probably function like microconidia or spermatia. When this fungus was cultivated in flasks on a homogeneous medium like oat-meal agar these bodies were again observed, though further development was lacking. Cultivated, however, on wheat grains with pieces of *Pinus*-branches, fully mature apothecia were obtained within three months. Although spermogonia-like bodies were always found, in a number of flasks, though treated in the same way, apothecia were lacking. The ascospores from the cultivated apothecia were identical with the original material.

Brefeld (2) who cultivated this fungus could only obtain a sterile yellow mycelium. Weir (18) observed small pycnidia with spores of 4 × 9μ. He called this stage *Dothichiza ferruginosa* Sacc. In my cultures this form was never seen. van Vloten (16) also obtained the perfect stage in vitro. In Denmark, however, Jørgensen (cit. van Vloten) did not succeed. The first author never found a *Dothichiza*-like stage. The difference in the formation of apothecia of this fungus may be explained by supposing that in some strains the property to form the perfect stage is failing.

Boyce (1) writes in his work on forest pathology: "Conidial stage unknown". We must conclude he also cultivated this organism.

In Finland Kujala (13) tried to grow the fungus. He obtained pycnidia on sterilized needles of pines with spores measuring (4—5) × (1.5—2)μ. These spores will be identical with the above mentioned spermatia. In nutrient solutions he only got a yeast-like mass of conidia, and could not obtain the perfect form. Ettlinger (7) who cultivated the fungus *Sclerophoma pityophila* (Sacc.) von Höhnel (syn. *Dothichiza ferruginosa* Sacc.) has moreover found that the growth of this fungus is markedly different from *Cenangium ferruginosum* Fries.

Summarizing we may conclude that this fungus sometimes developed apothecia but never a *Dothichiza*-form.

Encoelia fascicularis (Fr.) Karst., Myc. Fenn. **I**, p. 217, 1871.

After germination the one-celled ascospores soon became two-celled, while a germ tube was developing from both cells. On cherry decoction agar this fungus grew very rapidly. The mycelium was very delicate, showing a greyish-white colour gradually changing into white. Very small hyaline crystals were regularly found in the agar. After about 4 weeks mealy, slimy pustules developed which consisted of masses of more or less globular, hyaline microconidia measuring 1—2μ, cut off in immense numbers. These pustules are called sporodochia and consist of a central core of intertwined vertically branched conidiophores. To each conidiophore a microconidium is attached. On oat-meal agar the fungus grew excellently forming a mealy white mycelium gradually changing into yellow. After 2 months black pycnidia-like bodies were obtained; these bodies, which only possessed paraphyses, were sterile apothecia.

Various media were tested for the formation of apothecia, but only on wheat grains with pieces of *Fraxinus*-branch (ash) the fungus was able to form mature fructifications after 6 till 8 weeks (fig. 1). These fructifications measure 1 to 2.5 mm and possessed characteristic ascospores.

It was also possible to obtain cultures from apothecia collected on branches of poplars. The development in culture was quite the same as the above fungus isolated from ash. The apothecia were also identical with *E.fascicularis* (Fr.) Karst. In culture a few mature apothecia were obtained but never a *Dothichiza*-form was observed. Cultures of *Dothichiza populea* Sacc. et Briard only formed pycnidia in vitro, but never apothecia of a *Cenangium*-fungus.

In 1931 Foex (8) published a paper on a disease of poplars caused by *Cenangium populneum* (Pers.) Rehm. He supposed a connection with the fungus *Dothichiza populea* Sacc. et Briard. which has never been found in these studies.

Mollisia species. (no. 38 from stems of *Anthriscus*).

Studying the growth of some species in culture it was found that one of them was capable to form the perfect stage. On cherry decoction agar the mycelium was greyish-green with a black pigmentation of the agar. Apothecia only developed on malt agar and on sterilized stems of lupine or nettle after 8 weeks. From all my isolates this strain was the only one forming a number of fructifications, while other strains showed a greenish or greyish mycelium.

Brefeld (2) cultivated some species of this genus too, but he mentioned a few cases of formation of conidia and never of apothecia.

Pezicula cinnamomea (Fr.) Sacc., Michelia **II**, p. 332, 1881.

This Discomycete formed on cherry decoction agar a greyish, rather quickly growing mycelium with lightbrown aerial mycelium. After aging yellow hyphae gradually were seen, forming afterwards canary-yellow pustules in the agar. Though these pustules have been sectioned several times, they always proved to be immature and to consist of a dark stromatical tissue.

After a few transfers fully mature orange-yellow coloured apothecia were developed six month later. The ascospores were 2-till 4-celled and were measuring (26—28) × 9μ. In the same petri-dish also yellow-green slimy droplets were observed being acervuli of the fungus *Cryptosporiopsis* Bubák et Kabát. The spores from these bodies were 1- till 5-celled, measuring (28—39) × (11—14)μ, often possessing a transverse wall, and when aging of a light brown colour. Beside these forms one-celled, hyaline, bacilliformed cells, microconidia, measuring (4—9) × 1.5μ were noted. After identification the imperfect form proved to be the species *Cryptosporiopsis grisea* (Pers.) Petrak.

Wollenweber (21) as mentioned in the introduction could already cultivate apothecia. From a tumor on oak he isolated this fungus and obtained apothecia on oatmeal agar. Groves (10) never saw these fructifications in his cultures of *Alnus Peziculae*. According to the nomenclature in the sense of Groves this fungus is identical with *P. aurantiaca* Rehm.

Phialea acuum (Alb. et Schw. ex Fr.) Rehm, Krypt. Flora p. 717, 1896.

It was possible to cultivate this fungus on potato-dextrose agar forming here a bright white mycelium of very slow growth. After 7 weeks small black bodies were noted close to the glass wall. These bodies contained one-celled, hyaline spores, measuring about (7—8) × 4μ and strongly resembling those of the genus *Phoma*. In the same tube the very delicate, white, sometimes creamy-coloured apothecia were formed with asci and ascospores measuring (20—30) × 4μ and 4 × 2μ.

Sclerotinia sclerotiorum (Lib.) de Bary, Vergl. Morph. Pilze. 1884.

Cultures were obtained from sclerotia collected from diseased bean plants. Primarily these sclerotia were made to increase in flasks with grains of wheat. The obtained sclerotia were thoroughly cleaned and desinfected in sublimate 0.2 %, and then transferred to tubes with water agar.

During a long period these tubes were kept in the dark at low temperatures. After a few months very small, brown points developed from the sclerotia gradually showing fragile stipes. When these stipes measured about 2 cm the tubes were placed in daylight, but still at a low temperature (10—12°C.).

After 5 months fructification was obtained. The apothecia were long-stiped, brown, up till 4 cm in length. The hymenium of the fungus was about 3 mm, and lightbrown (fig. 2). One sclerotium could develop several apothecia. The asci were measuring 95—117 —9μ and the ascospores 10—12 (14) × 5μ.

When studying literature on the formation of apothecia by the *Sclerotiniaceae* we learn, that fertilization normally acts by means of microconidia or spermatia (DRAYTON). In this fungus such cells were never observed, while the formation of apothecia was easily secured. HENSON and VALLEAU (12) described similar methods a.o. cultivation on sterile sand or soil.

Summary.

The author made a study of the culture of *Discomycetes*. About fourty species were studied in vitro. An ascertaining of the conditions inducing the perfect stage was aimed at and as far as these fructifications developed they were studied.

From six of these fungi *viz.*, *Cenangium ferruginosum* Fr., *Encoelia fascicularis* (Fr.) Karst., *Mollisia* spec., *Pezicula cinnamomea* (Fr.) Sacc., *Phialea acuum* (Alb. en Schw. ex Fr.) Rehm and *Sclerotinia sclerotiorum* (Lib.) de Bary, apothecia were obtained in culture, the others failed to do so.

The writer is very much indebted to Miss Dr M. P. LÖHNIS and to Prof. Dr JOHA. WESTERDIJK for critical and valuable help in preparing the manuscript for publication.

Literature.

1. J. S. BOYCE, Forest Pathology. New York, 1948. – 2. O. BREFELD, Untersuchungen aus dem Gesammtgebiet der Mykologie. Ascomyceten II, Heft X. Münster i.W., 1891. – 3. R. W. G. DENNIS and E. M. WAKEFIELD, Transact. Brit. Myc. Soc. **29**, **141**, **1946**. – 4. F. L. DRAYTON, Mycologia **24**, **345**, **1932**. – 5. F. L. DRAYTON, Mycologia **26**, **46**, **1934**. – 6. F. L. DRAYTON, Mycologia **29**, **305**, **1937**. – 7. L. ETTLINGER, Ueber die Gattung *Crumenula* sensu REHM mit besonderer Berücksichtigung des *Crumenula*-Triebsterbens der *Pinus*-Arten. Thesis, Zürich, 1945. – 8. M. FOEX, Maladie des Peupliers déterminée par le *Cenangium populneum* (Pers.) Rehm (*Dothichiza populnea* Sacc.) Rapport Congres. Int. du Bois et de la Silviculture. Paris, 1931. – 9. M. J. F. GREGOR WILSON, Ann. of Botany **45**, **73** ,**1931**. – 10. J. W. GROVES, Mycologia **32**, **112**, **1940**. – 11. J. W. GROVES and F. L. DRAYTON, Mycologia **31**, **485**, **1939**. – 12. L. HENSON and W. D. VALLEAU, Phytopath. **30**, **869**, **1940**. – 13. V. KUJALA, Ueber die Kleinpilze der Koniferen in Finnland. Comm. Inst. Forest. Fenn. Helsinki. **1950**. – 14. M. NOBLE, Trans. Brit. Myc. Soc. **30**, **84**, **1948**. – 15. F. L. STEVENS, Phill. Agr. **19**, **491**, **1931**. –

16. H. VAN VLOTEN, *Rhabdocline pseudotsugae* Sydow., oorzaak eener ziekte van Douglasspar. Thesis, Wageningen. 1932. – 17. A. M. WATERMAN and E. K. CASH, Mycologia **42**, 374, 1950. – 18. J. R. WEIR, Phytopath. **11**, 166, 1921. – 19. H. H. WHETZEL, Farlowia **1** (3), 483, 1944. – 20. W. L. WHITE, Lloydia **4**, 153, 1941. – 21. H. W. WOLLENWEBER, Arb. Biol. Reichsanst. f. Land. u. Forstw. **22**, 521, 1939. – 22. H. W. WOLLENWEBER und O. A. REINKING, Die Fusarien, ihre Beschreibung, Schädwirkung und Bekämpfung. Berlin, 1935.

BOOKS.

B. STONEBRINK (Veterinary Health Board in Drenthe at Assen), Ontwikkeling van een voedingsbodem voor de primaire cultuur van *Mycobacterium tubercolosis* var. *bovis* (Development of a culture medium for the primary isolation of *Mycobacterium tuberculosis* var. *bovis*). Thesis Utrecht 1951.

Basing on the results of a study of literature a pigmented egg medium was composed. Various substances were added, the chief among which were pyruvic acid, phosphate buffer and active coal. In preliminary experiments this medium appeared to result in a gain of time of about 40%, when compared with the LOEWENSTEIN medium without glycerol. The growth itself moreover was much more abundant while colonies were very characteristic. During the investigation about 140 formulae for media were computed either by altering or leaving out various compounds or by adding new ones. The testing was carried out by means of inoculation with experimental strains in 6 decimal dilutions in 4-fold series. The inoculation was applied by pipette, while care was taken that an equal quantity of material was spread evenly on the culture medium.

During the first 3 weeks tubes were examined daily, later 2—3 times weekly. The following colony forms were observed:

1. smooth hemispheres.
2. R-forms: irregular, rough, white, rising steeply from the medium, later in form of bread crumbs or raspberries, measuring up to 3 mm.
3. S-forms: large, flat, spreading, granular, with a central knob, edges expanding thinly and irregular in outline, measuring up to 1.0 cm.
4. small, flat colonies, granular without central knob, measuring up to 1 mm.

The I-form appeared in case of strong inhibition. With less strong inhibition R- and S-forms occurred together, the stronger the inhibition the more R-forms. The IV-form was seen in media without pyruvic acid. These colony forms only appear when colonies have sufficient room, i.e., when the number per tube does not exceed 10 to 15.

The lag phase (here being the lapse of time between inoculation and the first growth noted with the naked eye) was inversely proportional to the logarithm of the actual number of colonies (in series of tubes inoculated with the same amount of bacteria).

A further inversely proportional ratio existed between the lag phase and the logarithm of the number of bacteria used as inoculum. The higher the pH the longer the latent phase.

The formula which finally appeared to be the most suitable for primary inoculation, was number **126**, having the following composition:

A.	Salt mixture	
	Pyruvic acid	1.0 g
	5 n NaOH to pH 6.5	
	KH_2PO_4	0.5 g
	Na_2HPO_4 to pH 6.5	
	Water to	75.0 ml
B.	Egg mixture	200.0 ml
C.	Dye mixture	
	Crystal violet	25.0 mg
	Malachite green	200.0 mg
	Water to	25.0 ml

Mix in the given order, fill tubes and let them solidify during 2 hours at 80—85° C. in an incubator saturated with water vapour.

Later the optimum of the phosphate buffer concentration appeared to lie somewhat higher. The amount of KH_2PO_4 was made up to 0.7 g, and the concentration of the dye mixture reduced to the half. This was the formula with the number **152**.

In primary isolations from material of various origin (mostly sputum) the lag phase appeared to lie between 8 and 35 days with an average of 20 days. When compared with the LOEWENSTEIN without glycerol the difference in the lag phase appeared to be 30%, the smaller the inoculum the larger the difference. This bore mostly on material from slaughtered tuberculous animals; with sputum, which generally contains fewer bacteria, this difference will undoubtely be still more marked.

Of all samples collected in the action for the combating of tuberculosis 5% were noted as positives.

Some methods were presented for the differentiation from the acid fast saprophytes. But for a few cases, no use has been made of experimental animals.

The method is almost completely based on the characteristics of the colonies.

Author's abstract.

Obituary Notice

HENDRIK CHRISTOFFEL JACOBSEN
1882—1952

On February 6th 1952 HENDRIK CHRISTOFFEL JACOBSEN died at Rotterdam in the age of seventy years. The name JACOBSEN may well remain without response from the majority of the members of the "Nederlandsche Vereeniging voor Microbiologie". Amongst the older members, however, there will be many who will remember the friendly personality of the deceased who was a regular attendant of the meetings, especially in the earlier years of the society. But only a few will realize that the death of JACOBSEN robbed microbiology of an exceptionally gifted votary.

JACOBSEN was born on January 29th 1882 at Rotterdam where he also passed the primary and secundary school („Hogere Burger School"). In 1899 he entered the "Polytechnische School" at Delft — the forerunner of the present Technical University — and in 1903 he took the final degree of "technoloog". For JACOBSEN, however, this did not mean a parting from his Alma Mater. Immediately after finishing his study he was appointed assistant to the professor of organic chemistry, the well-known Professor S. HOOGEWERFF. Yet one year later the event occurred which would prove decisive for JACOBSEN's further development as a scientist. He was invited by the Professor of microbiology M. W. Beijerinck to become his assistant. Jacobsen accepted the invitation; on September 1st 1904 he entered upon his new office and he remained in this post for almost 12 years.

This so unusually long duration of the academic assistantship at once betrays that something remarkable must have been going on in this period. This can be briefly summarized as an exceptionally fertile interaction between the microbiological master Beijerinck and a most capable collaborator.

There is no doubt that Jacobsen's rapid initiation in the science of microbiology is due to Beijerinck's sparkling genius, and to the stimulating example set by him. But Jacobsen had an extraordinarily receptive mind, and within a short period he developed into an independent worker, characterized on the one hand by great acumen, and on the other by an excellent command of laboratory techniques, both in the chemical and in the microbiological field.

Anyone who is acquainted with the book "Martinus Willem Beijerinck. His Life and his Work", and who has given due attention to the chapter "Beijerinck at work" will realize what it must have meant to this restless and passionate investigator to be able to profit by the cooperation of such a deliberate and devoted worker as Jacobsen has been. The more so since this collaboration covered almost one half of Beijerinck's academic career. In the first place Beijerinck could leave to Jacobsen a great part of his teaching duties, such as the preparation of demonstrations for the lectures, and the laboratory assistance to the students. But it is certain that Jacobsen by his painstaking and carefully devised experiments has also materially contributed to the success of Beijerinck's scientific work. In the publications from the Delft laboratory Jacobsen's part in the investigations has not always been duly acknowledged. The researches on the sulphur oxidizing bacteria, however, offer a characteristic example of this division of tasks. To Beijerinck goes the merit of the isolation and description of *Thiobacillus thioparus*, and in his publication the autotrophic character of this organism is already clearly indicated. It was, however, Jacobsen who, in two excellent papers published in "Folia Microbiologica", brought convincing experimental proof for the correctness of this assumption.

Jointly with Beijerinck Jacobsen published in 1908 the first observations on the influence of extremely low temperatures — 80 and 20 degrees on the KELVIN scale — on various microorganisms.

In another field JACOBSEN has broken virgin soil. In two papers which may almost be regarded as classics JACOBSEN laid down the results of his extensive investigations on the isolation and the metabolism of a special group of heterotrophic green algae. The experiments reported herein testify of JACOBSEN's at that time unrivalled mastership in handling the microbes in question.

In March 1916 JACOBSEN finally decided to leave his academic surroundings; he accepted the post of bacteriologist to the "N.V. Jurgens' Margarinefabrieken" at Oss, which industrial enterprise was later amalgamated into the "Unilever N.V.". JACOBSEN remained in the service of the latter concern till his death. In his remarkably well equipped laboratory at Rotterdam he has continued his applied microbiological work with great vigour; even his official retirement from active service in 1947 did hardly slacken his activities.

In 1918 two publications appeared on the rancidity of "vegetable" margarine and on means for its prevention; they testify that JACOBSEN from the start had dealt with a microbiological problem of great practical importance with outstanding success.

Since then not much of his activity has reached the public: the world concern in whose service JACOBSEN stood has thought it fit to silence him. Only a small group of initiated knows that, although publication of scientific results was denied to him, JACOBSEN has continued his work with unabating energy. In the first place all aspects of the deterioration of margarine and its prevention have received a more or less exhaustive treatment. In addition to this he made extensive studies on milk souring and the connected problem of aroma formation with the aid of "starters". In later years he made important contributions to the solution of microbiological problems connected with the preservation of meat and various meat products. The outcome of all these investigations is only to be found in the archives of the concern in question; if all this work had been published it would have been a lasting memorial to the microbiologist JACOBSEN.

It is only after JACOBSEN retired from active service that he found the opportunity to direct his attention also to some "sidelines" which apparently were not involved in the publication prohibition. In the years 1949—1951 three more papers appeared; especially that on the bactericidal action of cod liver oil contains several valuable observations.

The foregoing exposé may already prove that JACOBSEN was an extremely modest man; he was devoid of all personal aspirations and he found full satisfaction in the work itself. Apart from this he loved music, and thoroughly enjoyed good concerts; in his younger years he was a skilled pianist.

All who in some phase of their life have had the privilege of knowing JACOBSEN more intimately — and especially also his young collaborators to whom he has transferred his knowledge of and his love for the microbe world — will always keep his endearing personality in warm remembrance.

Delft, March 1952. A. J. KLUYVER.

(From the Veterinary Department of the National Institute for Public Health at Utrecht, from the Medical Inspection of Public Health at Utrecht and from the Veterinary Inspection of Public Health at the Hague).

ON THE OCCURRENCE OF *SALMONELLA BAREILLY* IN MAN AND ANIMALS AND THE MODE OF TRANSMISSION OF THE SALMONELLOSE CAUSED BY THIS SPECIES

by

A. CLARENBURG, G. D. HEMMES and W. WAGENVOORT

(Received February 27, 1952).

INTRODUCTION.

The knowledge of the genus *Salmonella* has extended in recent years. By means of examination of material of either human origin or derived from miscellaneous animals, species divergating in chemical action or antigenic structure from those known up till then have been recognized. Our knowledge, however, of the significance of such species in human or animal pathology, of their natural occurrence and their mode of spread, urgently needed for providing a basis for the control of disease, lagged far behind the quick rate at which discoveries were made in the laboratory.

By the assistance of many colleagues we had an opportunity in the Netherlands to make a number of observations which seem to us to provide a better understanding of the transmission of the salmonellose caused by *S. bareilly*.

S. bareilly was cultured for the first time in India by DABHOLKAR and BODMAN from blood of a patient in Bareilly (India), who suffered from a disease similar to typhoid fever. BRIDGES and SCOTT (1) identified this strain. In 1939 JUNGHERR and CLANCY (8) reported that they had isolated this germ from the liver of chicks. In a systematic investigation on the occurrence of Salmonella in patients in several hospitals SCHIFF and SAPHRA (12) detected in a butcher's wife with intestinal complaints besides *S. bovis morbificans* and *S. virchow* also *S. bareilly*. This was the first time this Salmonella had been noted in human material in U.S.A. In Europe RAUSS (10) at Budapest was the first to isolate *S. bareilly* from material of patients with spleen tumor and of one with a moderately high intermittent fever which had lasted 2 or 3 weeks. KAUFFMANN and SILBERSTEIN (9) detected *S. bareilly* in a patient suffering from gastro-enteritis in Puerto Rico. RUBIN, SCHERAGO and WEAVER (11) detected this germ in lymph glands of swine, EDWARDS and BRUNER (5) in material from fowl, swine and man. In 1943 SELIGMAN, SAPHRA and WASSERMANN (13 and 14) estimated that 1.8% of the salmonelloses in the population of the U.S.A. is caused by *S. bareilly*. Three years later they published a second series of observations presenting a percentage of the same order. HINSHAW, MCNEIL and TAYLOR (7) carried out extensive investigations of avian salmonelloses in California. 13 out of 291 infections in turkeys were caused by *S. bareilly*. 42% of the cultures from material of chickens were identified as *S. bareilly*. In these parts *S. bareilly* infections of economical importance occur yearly in poultry. The severest losses occur in the first month of animal life, but the disease occurs in animals of any age. Contaminated hatcheries may give rise to a spreading of the disease. They are of the opinion that human carriers in the latter may transmit the infection to the fowl. The risk of transmission of the disease by means of eggs they deem small but they point to the possibility of a contamination of personnel of poultry farms or poulterers, originating from poultry organs. SOLOWEY, SPAULDING and GOROSLINE (15) detected *S. bareilly* in egg powder and consider its consumption as a source of infection. The occurrence of *S. bareilly* in eggs and organs of poultry was corroborated by FELSENFELD, YOUNG and TAMA YOSHIMURA (6). Recently in the North of France BUTTIAUX, GAUMONT and MOREL (2 and 3) isolated *S. bareilly* from human blood, later from some chicks and from mesenterial lymph glands of swine. JOAN TAYLOR (16) identified as *S. bareilly* in 1949 8 strains from human material; CLARENBURG and ROEPKE (4) reported an increasing mortality of chicks in a number of poultry farms in the Netherlands during February and March 1951. Examination of organs and intestinal content revealed *S. bareilly* as the cause of infection. Desinfection of incubators with formaline vapour during the period of incubation was applied resulting in the absence of further complaints about day-old chicks.

Between 1948 and 1950 *S. bareilly* has been isolated from human material in 9 Dutch provinces; this species had never been detected before. This material bore on 24 isolated cases, while 8 times several inmates of a same house fell ill simultaneously or within a short period. In the course of 1951 a distinct increase in the number of human cases could be noted. While in January and February not a single case was reported, the increase occurred chiefly between the end of March and June 15.

In that period one or more persons fell ill in 38 families and also later the number of recognized cases was higher than in the corresponding months of preceding years. These cases recognized in the laboratory do not express the real morbidity. Now and then salmonellose bareilly takes a very mild course, not all patients will consult a physician, while in treating a gastro-enteritis the physician will not in every case have excreta or blood examined bacteriologically. Still the results of laboratory examination permit of the conclusion that morbidity has increased in 1951, as during 1948—1951 the indication for examination of material from these patients (with the exception of two cases) has not been influenced by special factors.

INVESTIGATION OF SOURCES OF INFECTION OF HUMAN CASES.

Apart from contamination clearly due to human contact, the endeavour to reveal the source of infection yielded hardly any valuable data in 1948—1950.

The above mentioned results of CLARENBURG and ROEPKE, obtained in their investigation of chicks in the first quarter of 1951, induced us to consider any occurrence of contact with fowl in human cases and to interrogate also patients which had proved positive in 1948—1950 as to this point. This investigation, however, could not be undertaken on an extensive scale before the beginning of June.

Live chicks.

Whenever, before the initial symptoms, the patients had been in contact with birds inquiries were made concerning the mode of this contact and the occurrence of any abnormal mortality among the birds. If dead birds were present these were examined, while moreover feces in the poultry houses was collected. In the course of 1951 feces from the following avian species has been examined: chick, hen, duck, crow, homing-pigeon, wood-pigeon, finch, canary and Japanese nightingale. When a great number of poultry was concerned preliminarily 5 or 6 samples of feces were tested. In this way *S. bareilly* was successfully isolated from chick feces and from dead chicks owned by 9 patients affected with salmonellose bareilly. Feces of other birds belonging to the patients proved negative. A short survey of these nine cases is presented.

1. A poultry farmer received 102 day-old chicks of one hatchery on April 4. His wife who took care of the chicks fell ill on April 7; in her feces of April 12 *S. bareilly* was detected. Among the chicks 10 died fairly soon. From the liver and intestinal content of a chick slaughtered on April 28 *S. bareilly* was isolated. A mixture of chick feces sampled on June 18 proved negative.
2. On April 10 about 100 day-old chicks were delivered by a commission-agent to a farmer. The farmer's wife had been round the chicks but according to her saying had not touched them. She fell ill on the night of 13—14 April and from her feces of April 30 *S. bareilly* was isolated as well as from a mixture of chick feces, taken June 15th. In the week following April 10 two of the chicks died; other customers of the commission-agent noted a higher mortality. The hatchery which had supplied the chicks has appeared to be contaminated with *S. bareilly*.
3. On March 8 a farmer received from two hatcheries altogether some 500 one day chicks. In the course of April part of these chicks was transferred elsewhere. A neighbour who was daily in the immediate neighbourhood of these chicks fell ill on April 28; in his feces of May 4 *S. bareilly* was detected. On June 16 *S. bareilly* was isolated from chick feces. Among these chicks which could move about over a large area of land hardly any mortality had occurred. Also among other customers of one of these hatcheries which had supplied the chicks, cases of disease have been ascertained which are described under 7 and 9.
4. A person who for domestic use keeps a small number of chickens received on April 18 about 10 day-old chicks from a hatchery, while a hen of his own brooded eggs, 7 of which hatched on April 15. On June 11 his wife fell ill and from her feces of circ. June 20 *S. bareilly* was cultivated, as well as from a mixture of hen and chick feces collected on June 6.
5. On April 18 a hatchery sold 20 day-old chicks. The new proprietor fell ill on May 22, his feces of May 25 and the feces of his chicks sampled on June 18 contained *S. bareilly*. The chicks had not shown any symptoms of disease, in contrast with those of another customer of the same hatchery.
6. After having unpacked in the morning of April 21 a number of day-old chicks a farmer fell ill in the evening of the following day. In his feces of May 1 *S. bareilly* was detected. Shortly afterwards the same germ was isolated from the feces of his chicks. The chicks had a spacious run; no mortality occurred among them.

7. On May 12 the son, aged 12, of a factoryworker fell ill, his feces of May 28 contained *S. bareilly*. He had taken care of 35 chicks which were kept in a small shed and which had been derived as day-old chicks from one of the hatcheries, mentioned in 3. After a short period daily about one or more of the chicks died. In the organs of a chick having died on June 9 and of two on June 13, *S. bareilly* was detected, as well as in a mixture of chick feces, gathered on June 12.
8. A boy, aged 11, fell ill on May 4; *S. bareilly* was cultivated from his feces of May 12. April 17 the family had bought 10 day-old chicks in a store. Among the animals mortality occurred. The last remaining live one was sent to the laboratory on June 19. From the intestinal content of this chick as well as from the feces collected in the shed, *S. bareilly* was isolated. On July 7 chicks from the same store proved positive for *S. bareilly*.
9. From one of the hatcheries, mentioned in 3, a boy bought 5 cockerels. One of these died by accident, among the others two were sickly and did not thrive. His father had handled these when killing them. Soon afterwards on April 27 the father fell ill, his feces of May 3 contained *S. bareilly* as well as the mixed feces of the remaining cockerels. The latter animals have been killed; in the organs of one of them *S. bareilly* was detected. On June 26 this germ was cultivated from feces of two other batches of cockerels, derived from the same hatchery.

These observations point to the fact that contact with *S. bareilly* excreting chicks may cause infection in man. In some cases the symptoms of disease occurred soon after the receipt of the day-old chicks, while repeatedly these chicks appeared to have been derived from contaminated farms. Not a single indication was found which might lend support to the assumption of HINSHAW e.a. that human carriers would transmit the disease among the chicks. A very narrow contact with chicks had existed for some patients, but it occurred as well that patients denied any handling of the chicks, having merely been in their neighbourhood. It may be that these patients did not remember exactly what had happened, or that they have been infected by the ingestion of contaminated matters or by touching contaminated objects.

Besides these cases where examination of feces had clearly proved chicks as source of infection, there had occurred in 1951 still a number of cases where a narrow contact with chicks had existed shortly before the onset of disease, but where no *S. bareilly* had been detected in the sample of chicks' feces.

We further describe four cases of disease, some of which of earlier date, where examination of chick feces is lacking, but in which as seen later, chicks surely may be considered as a probable source of contamination.

On March 21, 1949 a hatchery had supplied day-old chicks. The purchaser in view of the cold weather kept them in the same room as his baby of 7 months. The parents made the baby pet the chicks. March 24 the baby fell ill; from her feces of April 12 *S. bareilly* was isolated.

Three days after having received on March 8, 1950 500 chicks from the same hatchery as above, the purchaser and his wife, who both had handled the chicks contracted diarrhea. Their only child, aged 11 months, who in that period had her abode in the upper stock and whom was not taken care of by other persons fell ill on March 13. From her feces of March 13 and blood of March 20 *S. bareilly* was cultivated. Among the chicks no mortality occurred. The parents in their own case had not consulted a physician so that their presumptive transmission of the disease could not be confirmed by bacterial examination. Their feces of April 18, 1950 did not contain *S. bareilly*.

A patient who fell ill in the evening of April 16, 1951 (feces of April 19 *S. bareilly* positive) had spent the day before elsewhere with a member of his family who had received 35 day-old chicks on April 10. These animals which had been kept in a narrow shed had all died in the course of a week. During our visit no remainders either of chicks or their feces were present any more.

A repairer of incubators in parts where many hatcheries were contaminated, suffered from diarrhea in the period of 1—14 April 1951 and in the first week of May. A third attack began on June 8. On June 17 a sample of the feces was sent in for the first time and *S. bareilly* was cultivated from them. This patient had much professional contacts with chick feces.

In 1951 in the latter half of June, so 5 weeks after the brooding season, the number of human cases decreased quickly. As the level remained low during some months, the risk of infection appeared to have diminished. Should this merely be ascribed to a lesser handling of the chicks than in their earlier life or would a decrease in number of germ excreters have occurred? To answer this question in the first half of August a greater number of samples of feces than initially was taken from the night sheds of the contaminated poultry farms, cited above in 2, 3, 4, 5 and 6 and moreover from one other poultry farm that had been found contaminated in June. In none of these samples *S. bareilly* was detected. The conclusion seems warranted that after the first months the number of excreters among chicks had markedly decreased. In this connection we point to the above cited communication of HINSHAW e.a., that in California *S. bareilly* causes the severest losses among poultry in their early age.

We further point to the fact that apparently the mortality of chicks from contaminated farms varies greatly. The keeping of many contaminated animals in an overcrowded area appears to augment the death rate. An absence of mortality does by no means signify freedom from *S. bareilly*. In our opinion combating measures should not be limited to those hatcheries where customers complain of mortality among the chicks.

Slaughtered chicks.

HINSHAW e.a. expressed the view that poultry farmers and poulterers might become infected by the handling of organs of contaminated animals. We have met with two cases where such a mode of contamination could not be excluded. A patient bought on June 16, 1951 for an inmate of the same house in a poulterer's store a chicken ready for roasting. In the morning of June 20 he suddenly fell ill, his feces of June 26 contained *S. bareilly*. The other case bore on a cook who daily purchases live chickens, slaughters these himself and prepares them. He fell ill on September 25, 1951, in his feces of October 15 *S. bareilly* was detected.

Any infection which might be ascribed to the eating of cooked chicken did not come to our notice.

Other animals.

In connection with the findings of BUTTIAUX e.a. who could culture from mesenterial lymph glands of 3 swine *S. bareilly* the possibility has to be kept in mind that meats containing swine mesenterium might form a source of contamination. As far as we have interrogated the patients in this respect, no cases have been noted where meats might be suspected. In the Netherlands never up till now *S. bareilly* has been detected in the mesenterial lymph glands of swine.

During a mass investigation of rats from various regions of this country in view of the detection of another *Salmonella*, on October 30, 1951 unexpectedly one of them was found contaminated with *S. bareilly*. This rat came from a neighbourhood where much poultry was kept. Although in that region no human cases have as yet been ascertained the possibility has to be considered that this salmonellose might be transmitted by rats. A contamination of chicken food by rats has to be kept in mind. An examination of 6 samples of chicken food used in 4 farms July 28, 1951 proved negative.

Among the deer who were kept on the ground of a zoological garden 3 died on February 5 and 13 and March 7, 1949 respectively. On post-mortem examination they showed signs of a haemorrhagic gastro-enteritis. The last victim which since February 22 had excreted a thin bloody feces has been examined bacteriologically; from the intestine *S. bareilly* has been isolated. On bordering ground a deer died on April 18 which had been suffering from diarrhea a few days before his death. Human cases connected with deer did not come to our notice.

Merely in a few cases other animals than birds in the environment of patients have been examined, so the feces of some dogs and a rat which had been found dead on the farmyard of a patient. In this material no *S. bareilly* has been detected.

Transmission from man to man.

It could repeatedly be ascertained that persons affected with salmonellose bareilly transmitted their disease to inmates of their house, more especially to those who nursed them. A striking example is furnished by an aged person with a severe diarrhea who had initially for two days been nursed by a housemate, who then fell ill himself, the following two days by another person who also fell ill. He then was transported to a family elsewhere where the person who took care of him after 2½ days showed the same symptoms. Subsequently he was hospitalized. *S. bareilly* was cultivated from the feces of these four patients.

Also in hospitals spread of infection may occur as is shown by the following three observations.

In an infants' ward salmonellose bareilly set on simultaneously in three children; the infection was most probably due to a positive child in the same ward.

One of the patients in a ward for infectious diseases was suffering from typhoid fever. In the course of her disease *S. bareilly* could be isolated from her feces. Two patients in this same department were affected with this salmonellose.

In another hospital a patient had for 4 weeks been nursed for epididymitis tuberculosa, when suddenly a gastro-enteritis set on; in his feces *S. bareilly* was detected. In order to trace the source of infection, the excreta of the patients of the same ward were examined. One of this group of persons without symptoms of salmonellose appeared to excrete *S. bareilly*. This person, who suffered from commotio cerebri had, however, been admitted after the onset of gastro-enteritis in the first patient, consequently he was not the source of infection but he himself had probably been infected in the ward.

In the following two cases where the disease had broken out explosively food seemed to have been contaminated by manual contact.

In the evening of March 19, 1949 a group of 45 persons had eaten cooked macaroni and currant-juice, which had been served soon after its preparation by a person who took care of pigs and poultry. These persons remained free from complaints. The remainder of the macaroni which had been stored during the night in the heated kitchen has been consumed March 20 at 7.30 a.m. by ten young men out of a group of 12. Two who had abstained from eating this food remained healthy, but the others contracted gastro-enteritis, the first in the evening of March 20, the others March 21. In three of them who had eaten but little of the macaroni, the disease ran a mild course. The feces of two patients has been examined bacteriologically and in both *S. bareilly* was detected.

On May 2, 1951 early in the morning in a pastry shop the baked paste consisting of eggs, sugar and flour for 60 pastries was ready. A paste consisting of milk which had been boiled with cream powder and sugar had still to be added. The baker's man directly after his arrival made up 30 to 35 of these pastries, packed them and delivered them to two families. 9 out of 17 persons who had eaten of them fell ill. In their feces *S. bareilly* was detected as well as in the feces of the 8 other persons who did not show any clinical symptoms. In the further part of the day the baker's man has merely delivered bakery ware and has not been active in the bakery. The remaining 25 or 30 pastries of the same batch have not given rise to any complaints. It is thus improbable that some constituent of the pastries, *viz.*, the eggs would have given rise to the infection. The observed facts would be in better agreement with a contamination during the adding of the paste. Examination proved the baker's man, although he did not feel ill, to excrete *S. bareilly*. He had not eaten of the pastries in question.

Hens' eggs.

The question rises whether the use of hens' eggs might give rise to salmonellose bareilly in man. A germinative contamination of hens' eggs may be conceived like it has been observed for *S. typhi murium* in ducks' eggs. Also the shell may be soiled by *S. bareilly* containing feces while under certain conditions *S. bareilly* occurring on the exterior surface of the shell may penetrate into the interior of the egg. We have made a preliminary attempt to solve this question by examining eggs lain by hens born in 1951, which from their birth had abided uninterruptedly in poultry farms where chicks had been found to be infected with *S. bareilly*. From such poultry farms 481 eggs of young hens were examined in September, October and November. The result proved negative. We intend to continue the investigation concerning the occurrence of *S. bareilly* in adult chickens, using the agglutination test and the examination of feces and eggs. We intend to examine any chickens which may prove positive more especially as to infection of the ovary.

The number of hens' eggs consumed as such or worked up in victuals is very large in this country. The method of working up is often not adequate to warrant killing of the salmonellae which may be present in the egg.

The number of human cases recognized as salmonellose bareilly in which the disease has set on without any previous contact with chicks or with known human excreters of germs is fairly small, *viz.*, 22 in the period between January 1 and December 1, 1951. In some of these the consumption of any eggs in the latter weeks preceding the illness was denied very strongly, but in most cases the patients had done so. In a few cases there was a special indication that the consumption of an egg might have been the cause of the infection.

First of all the already mentioned patient with epididymitis. He received from his visitors exclusively oranges and ate further only the hospital food. In the evening of June 8 and June 9 he had eaten a soft-boiled egg. On June 10 his temperature rose suddenly up to 40° C. and he contracted a severe gastro-enteritis while persons in his environment remained free.

A man, 22 years of age, consumed on November 3, 1951 at 11 a.m. and on November 5 at 6.15 a.m. a beaten-up egg. He fell ill on November 5 at 4 p.m. (feces of November 10 positive for *S. bareilly*). Any other cause of this infection than the beaten-up egg could not be made probable.

Except the case mentioned of an infection through pastries, where eggs could scarcely be considered as the causal agent, the consumption of victuals or drinks with eggs as a constituent never gave rise to a mass infection such as previously has come repeatedly to our notice after the eating of contaminated ducks' eggs. Further investigation along the lines mentioned seems necessary to us in order to elucidate in how far eggs might be a possible source of infection.

In a number of human cases we were left in the dark as to the source of infection. Other modes of infection might exist; here we have in mind a spread by chick feces which would be hard to ascertain.

Meanwhile the combating of the disease in chicks has been taken to hand by means of desinfection of the incubators and the incubated eggs with formaline vapour.

A paper containing more extensive statistical and clinical data will be published by G. D. HEMMES in the journal ,,Geneeskundige Gids".

Summary.

In the Netherlands salmonellose due to *S. bareilly* was noted for the first time in 1948, initially only in man, but in the beginning of 1951 also in chicks. Between the end of March and the latter half of June the number of human cases has distinctly increased. Nine patients appeared to have had a recent contact with chicks which excreted *S. bareilly* or harboured this germ in their organs. The percentage of excreters of germs among chicks decreased after the first months. 481 eggs lain in autumn by young hens from poultry farms which had been found contaminated in spring 1951 did not contain *S. bareilly*. Also a diseased person can spread the disease either by direct contact or by contaminating foodstuffs. In the course of our investigation *S. bareilly* has been incidentally cultivated from a rat and from material originating from a deer.

Literature.

1. R. F. BRIDGES and W. H. SCOTT, J. Royal Army Med. Corps **56,** 241, 1931. – 2. P. BUTTIAUX, R. GAUMONT and P. MOREL, Ann. Inst. Pasteur de Lille **3,** 140, 1950. – 3. P. BUTTIAUX, R. GAUMONT and P. MOREL, Ann. Inst. Pasteur **81,** 236, 1951. – 4. A. CLARENBURG en W. J. ROEPKE, Tijdschr. Diergeneesk. **77,** 174, 1952. – 5. P. R. EDWARDS and D. W. BRUNER, J. inf. Dis. **72,** 58, 1943. – 6. O. FELSENFELD, V. M. YOUNG and T. YOSHIMURA, J. Amer. Vet. Med. Ass. **66,** 17, 1950. – 7. W. R. HINSHAW, E. McNEIL and T. J. TAYLOR, Amer. J. of Hyg. **40,** 264, 1944. – 8. E. JUNGHERR and C. F. CLANCY, J. inf. Dis. **64,** 1, 1939. – 9. F. KAUFMANN und W. SILBERSTEIN, Zentralbl. f. Bakt. **132,** 431, 1934. – 10. K. RAUSS, Zentralbl. f. Bakt. **147,** 253, 1941. – 11. H. L. RUBIN, M. SCHERAGO and R. H. WEAVER, Amer. J. Hyg. **36,** 43, 1942. – 12. F. SCHIFF and I. SAPHRA, J. inf. Dis. **66,** 2, 1940. – 13. E. SELIGMANN, I. SAPHRA and M. WASSERMANN, Amer. J. Hyg. **38,** 226, 1943. – 14. E. SELIGMANN, I. SAPHRA and M. WASSERMANN, J. of Imm. **54,** 69, 1946. – 15. M. SOLOWEY, E. H. SPAULDING and H. E. GOROSLINE, Food Res. **11,** 380, 1946. – 16. JOAN TAYLOR, Brit. Med. Bull. **7,** 163, 1951.

(From the Laboratorium voor de Gezondheidsleer der Universiteit, A'dam).

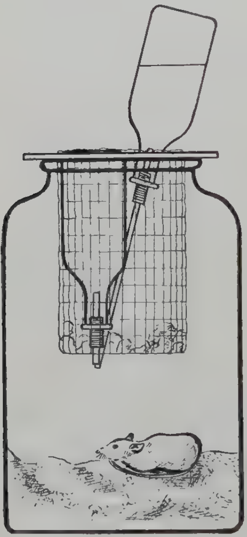
A CHEAP AND SAFE METHOD OF KEEPING MICE

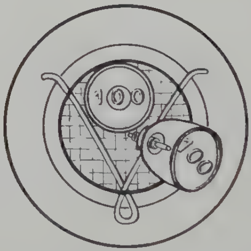
by

F. DEKKING

(Received February 5, 1952).

In this laboratory mice are bred and kept in wide-mouthed, 3 l glass jars, as used for the herring-packing industry. The mouth of this jar is completely occupied by a wire-mesh basket which serves as food (pellets) container and at the same time makes escape of mice impossible. The basket is supported on the rim of the jar by a piece of sturdy wire, shaped in such a way that it allows the placing of the drinking bottle in either of two positions: in, or on top of the basket. The first position is used in breeding mice: it saves space, but the bottle can be contaminated on the outside by climbing mice (thus creating the possibility of contamination of the hands when changing bottles, with the risk of cross infections), so with infected animals the second position is used. The bottles have a capacity of 100 ml (about three day's supply for four mice) and are not refilled without previous sterilization. The glass tube, surrounded by a piece of rubber tube, fits closely in the mouth of the bottle. Baskets and jars are easy to clean and sterilize. Sterilized sawdust is used as a bedding.





(From the Laboratory for Tropical Hygiene of the State University, Leiden).

ON THE INDUCTION OF VI-ANTIGEN FORMATION IN A STRAIN OF *SALMONELLA TYPHI* FREE OF VI-ANTIGEN

by

HANS L. BOOY and **HENDRIK L. WOLFF**

(Received December 19, 1951).

The experiments described in this paper were inspired by the investigations on capsule formation in Pneumococci carried out by GRIFFITH (5) and by AVERY and his school (1). These authors had found that capsule-free Pneumococci may be induced to form capsules by a treatment with extracts from encapsuled ones. It was further found that this was not due to the actual substance the capsules consist of, which is a polysaccharide, but to a polymer of desoxyribose nucleic acid, i.e. to a substance we know to occur inter alia in the nuclei of higher organisms.

The results obtained with these Pneumococci suggested to us the possibility that a similar change might be induced in typhoid bacteria lacking Vi-antigen, *viz.*, that they might be induced to give rise to an offspring possessing the faculty to produce this antigen. The effect we had in mind should not be confused with a change of the W character of a strain into the V character. Indeniably such a change might lead to a similar result, as the so-called W strains are usually able to produce some (incomplete) Vi-antigen. Growing strains in plate culture reveals the reason of this, for then the bulk of W colonies appears to be mixed with a few typical V ones. When one of the W colonies in its turn is sown out, this phenomenon reappears. In order to eliminate the risk of a change of this kind, we had to start from a strain of typhoid bacteria which was known to lack completely the production of any V colonies. Our laboratory possessed one such strain; it has been registered as H 901. As we have not yet succeeded in isolating Vi-antigen-free strains among those in which this substance is present, we are unable to tell from which phage-type our strain H 901 may have been derived.

Following AVERY in technique we performed our experiments in vitro. The strain that we chose as Vi-antigen producer (Vi 643) differed in its biochemical properties from our Vi-antigen-free H 901, in as far that it did not split xylose. According to CRAIGIE's classification (2, 3) based on the phage-typing, it belongs to the type D 6.

We worked also with a strain of *Salmonella Ballerup*, a species more recently classified by certain scientists as *Escherichia*. It differs considerably from *S.typhi*, but corresponds with the latter in its faculty to produce a Vi-antigen. We have tried to classify it by means of the Vi-phage II, but we did not succeed in obtaining plaques. It also proved impossible to adapt the Vi-phage II to this strain.

TECHNICAL DETAILS.

I. Indicator plates for xylose:

agar	3	g
peptone (bacto)	1	g
xylose	1	g
NaCl	0.5	g
bromthymolblue, resp. phenol red	0.04	g
water	100	ml

The liquid is sterilized by heating to 105°C., and just enough NaOH or HCl is added to obtain the reversion of the colour to blue, resp. to red.

II. Dorset's culture medium:
1 part of saline (0.9 %)
3 parts of egg.

On the first day heated for 2 hours to 85°C.; on the second day for 1 hour to 80°C.

III. For growing large amounts of typhoid bacteria the solution recommended by FREEMAN e.a. (4) was used.

IV. Phage-typing according to CRAIGIE (cf. LIE KIAN JOE (6)). Dr LIE supplied us with the phages which had originally been prepared by Dr FELIX.

V. The serological test for Vi-antigen was carried out with two different kinds of sera, *viz.*, 1. rabbit antiserum against the Ballerup strain, and 2. saturated sera against Ty II Bhatnagar or similar Vi-antigen-carrying typhoid strains; the saturation was obtained by means of the strain H 901, the strain we had started with.

VI. The strains of bacteria used in our experiments were:

a. Vi 643; a strain of *S.typhi* obtained from Djakarta and belonging in CRAIGIE's classification to the type D 6; it does not split xylose;
b. *S.typhi* II;
c. *S.typhi* Bhatnagar;
d. the *S.typhi* strain known as H 901;
e. the *S. typhi* strain R 2, supplied by Dr CLARENBURG, Utrecht;
f. *S.Ballerup*;

Before we started our experiments it was necessary to ascertain whether the strain Vi 643, which itself is unable to split xylose, might not give rise to an offspring producing this reaction. To this end use was made of indicator plates for xylose (I). The result was negative; we have never been able to detect on these plates a single colony attacking xylose. It is perhaps worth while to add that we neither succeeded in obtaining from our strains possessing this faculty, variants that had lost it. Therefore, although we can not prove that none of such variants may arise, it seems very improbable that they would have been formed during our experiments in sufficient number to affect the results.

We attacked the problem in two essentially different ways: 1. we studied the effect of killed bacteria and of the products of the bacterial metabolism. 2. we worked with living bacteria, growing two traceably different strains in a mixed culture and by testing the offspring of its constituents it was ascertained whether an exchange of antigens had taken place.

As to the experiments belonging to the first group, those that were carried out either with liquid or with solid culture media free of bacteria, were unsuccessful, but by using solid culture media in which dead Vi-antigen-producing bacteria were present, we succeeded in obtaining from the strain H 901 a great number of Vi-antigen-producing descendants. This result was reached in the following way. Vi-strains (i.e. strains producing Vi-antigen) were grown for 16 tot 20 hours in FREEMAN's culture solution, the best results being obtained with *S.Ballerup*. At the end of that period the flask containing the culture was heated to 56°C., and kept for two hours at that temperature, stirring the liquid now and then in order to prevent the occurrence of any differences in temperature which might lead to the escaping of bacteria from its lethal action. Then the culture was centrifuged, and the bacterial sediment washed three times with a physiological salt solution, part of the material being sown out in order to ascertain the absence of any living bacteria. For the experiment itself 800 mg of the humid bacterial sediment was mixed with 80 ml of a 2.5 % nutrient-agar heated to a temperature of 45°C. This mixture was poured out in dishes and on these plates a culture of *S.typhi* H 901 was smeared out, which had been diluted so far that the colonies could develop separately. After an incubation time of 16 to 20 hours at a temperature of 37°C. the colonies were tested one by one for the presence of Vi-antigen by means of the slide agglutination. Positive colonies were directly transferred to normal nutrient broth containing agar. These cultures too were kept at a temperature of 37°C., and were subjected the following day to the test for Vi-antigen. In this way we succeeded in isolating strains that, even after 18 transfers, still contained Vi-antigen. A quantitative estimation showed that the Vi-antigen of this fresh strain is not identical with the native one found in *S.typhi* (cf. Table I and II and the discussion of the results reported).

As stated above, also by another method we could induce *S.typhi* H 901 to produce strains able to agglutinate anti-Vi sera, *viz.*, by mixing this strain, which possesses the faculty to split xylose, with the Vi-strain 643, which lacks this faculty. This mixture was cultivated for 4 to 20 hours at a temperature of 37°C., and then smeared out on indicator plates for xylose. As the H 901 colonies are yellow and surrounded moreover by a yellow rim, they stand out clearly against the blue- or may be red background, at least so long as the incubation time does not exceed a certain limit; if it does, the whole plate assumes a yellow tint, and then the H 901 colonies are no longer distinguishable. These yellow colonies were tested by means of slide agglutination for the presence of Vi-antigen, and quite a number of them proved to be provided with this substance. So also in this way we succeeded in obtaining strains of *S.typhi* provided with the faculty to produce some kind of Vi-antigen. We controlled the behaviour of such a strain during a long series of transfers, and here too, at least when the strain was grown on a suitable medium, *viz.*, Dorset's egg-agar, the faculty to produce the antigen proved to be retained. The faculty to split xylose was also preserved.

We selected two strains for further study, *viz.*,

1. H 901 A, one of the strains obtained by means of the first method, i.e. by cultivating the mother strain H 901 on bouillon agar mixed with a killed culture of *S.Ballerup*, and
2. H 901 B, one of the strains obtained by means of the second method, i.e. by growing H 901 in a mixed culture with Vi 643.

We did not succeed in determining the place these strains would occupy on account of their behaviour to various phages in CRAIGIE's classification, and our attempts to adapt phage II to them were neither successful. Brought together with the phages I and IV there proved to be no lysis.

The results of our other experiments with these strains are summarized in the Tables 1 and 2.

TABLE 1.

Agglutination titer of various strains of *S.typhi* and of *S.Ballerup* with the serum against *S.Ballerup*; 1) directly; 2) after saturation of the serum with H 901 A; 3) after saturation of the serum with H 901 B.

name of the strain	1)	2)	3)
S.typhi H 901	—	—	—
S.typhi H 901 A (14th transfer)	128	—	—
S.typhy H 901 B (12th transfer)	64	—	—
S.typhi Bhatnagar	256	128	128
S.typhi II	512	128	128
S.Ballerup	8000	1024	8000

TABLE 2.

Agglutination titer of the same strains with the sera against H 901 A (1) and H 901 B (3), respectively after saturation of these sera with H 901 (2 and 4).

name of the strain	1)	2)	3)	4)
S.typhi H 901	8000	—	8000	—
S.typhi H 901 A	8000	512	8000	64
S.typhi H 901 B	8000	128	8000	128
S.typhi Bhatnagar	32	32	32	32
S.typhi II	8000	128	8000	128
S.Ballerup	512	512	64	64

DISCUSSION.

In our opinion it is fitting to conclude from the data reported that:

A. a) It is possible to obtain from Vi-antigen-free strains of *S.typhi* an offspring that forms Vi-antigen and retains this faculty through repeated transfer.
   b) It is possible with these strains to induce Vi antibodies in rabbits.

B. This change can not be ascribed to an absorption of Vi-antigen because
   a) in the case of H 901 A the Vi-antigen that was present in the culture of *S.Ballerup*, must have been destroyed by the heating to 56°C.;
   b) in the case of H 901 B the amount of Vi-antigen is far larger than that which might have been taken over from the strain Vi 643; and
   c) in both cases the Vi-antigen is still present after a considerable number of transfers.

C. There are strong indications for a complex character of the native Vi-antigen and for the absence of some of the latter's constituent factors in the Vi-antigens of the strains H 901 A and H 901 B;
   a) these strains appear to differ from the earlier known strains of typhoid bacteria: 1. by a lower agglutination titer (cf. Table 1) and 2. by the fact that the type of phage by which they would be dissolved, could not be determined;
   b) it proved impossible to saturate the ordinary Vi sera either with H 901 A or with H 901 B (cf. Table 2);
   c) Contrary to this it proved possible to induce by means of these strains the formation of incomplete Vi agglutinins (cf. Table 2).

D. The strain H. 901 A obtained from *S.Ballerup* produces not only the (incomplete) Vi-antigen but also some other antigens. This is reported in both tables.

The fact that H 901 B combines the faculty to split xylose, which apparently is not an easily transmissible one, with the possession of an incomplete instead of a complete Vi-antigen apparently furnishes proof that it must have been obtained from H 901 under the influence of Vi 643, and not from the latter under the influence of H 901.

In the introductory paragraph we mentioned the experiments of AVERY c.s. (1). These investigators had succeeded in giving (or perhaps restoring) to Pneumococci lacking capsules, the faculty to produce these. They obtained this result by means of extracts made from cultures of encapsuled Pneumococci, and these extracts proved to contain no polysaccharides, which means that they lacked the substance of which the capsules consist. Whether our results and theirs rest on the same principle, we must leave open. Nor are we in a position to decide whether or not hereditary factors are involved, as has been suggested by several investigators.

Summary.

We succeeded in providing Vi-antigen-free typhoid bacteria with an incomplete Vi-antigen. This result was obtained in two different ways:

1°. by growing these Salmonellas on plates that contained killed Vi-antigen-producing bacteria, and

2°. by growing a mixture of a Vi-antigen-free and a Vi-antigen-containing strain in a liquid medium.

In two strains, each of them obtained in a different way, the character of the Vi-antigen was studied. They proved to be different from the native Vi-antigen and also from each other.

It appeared that even after several (up to 18) transfers the Vi-antigen was still present. The new strains appeared to be able to induce the formation of Vi-antibodies.

References.

1. O. T. AVERY, G. M. MAC LEAD and M. MC CARTY, J. Exp. Med. **79**, 137, 1944. – 2. J. CRAIGIE and C. H. YEN, Canad. Publ. Health J. **29**, 448, 484, 1938. – 3. J. CRAIGIE and A. FELIX, Lancet **1947**, 823. – 4. G. G. FREEMAN, Biochem. J. **37**, 601, 1943. – 5. F. GRIFFITH, J. Hyg. **27**, 113, 1928. – 6. LIE KIAN JOE, Documenta Neerlandica et Indonesica de Morbis Tropicis **1**, 145, 1949.

(Microbiological Chemistry Section, National Chemical Laboratory, Council
for Scientific and Industrial Research, Pretoria, and Institute for
Pathology, University of Pretoria, Pretoria).

AN OBLIGATE HALOPHILIC BACTERIUM FROM SOLAR SALT

by

C. J. P. SPRUIT[1]) and A. PIJPER

(Received January 16, 1952).

During an investigation of a sample of so-called "solar salt", originating from Cape Cross, South West Africa, we isolated an obligate halophilic bacterium, possessing unusual properties.

Our sample of salt showed a distinct red colour. Its microbiological investigation was requested because salt of the same origin was reported to cause a red discoloration of animal products treated with it for purposes of preservation.

Cases of similar nature have been reported repeatedly and red discoloration is well known in all countries from where salted animal products are exported to warmer climates. As early as 1922, HARRISON and KENNEDY (1) devoted an extensive study to this red discoloration and traced it to the use of salt, obtained by the evaporation of sea water in tropical regions. The literature shows that red bacteria have repeatedly been isolated from such sources or from material treated with such salts. The description of their properties leaves little doubt that most of them are closely related. Unfortunately, their obligate halophilic nature offers obstacles in their examination with the more usual microbiological techniques. They have not yet been described in sufficient detail for their taxonomic position to be fixed satisfactorily. We were attracted to their study mainly because our strain possesses curious morphological properties, a description of which is the main aim of this paper.

---

[1]) Present address: Laboratorium voor Plantenphysiologisch Onderzoek der Landbouwhoogeschool, Wageningen, Netherlands.

METHOD OF ISOLATION; CULTURAL CHARACTERISTICS.

Lumps of the red salt were introduced into Erlenmeyer flasks containing pieces of stockfish (hake, *Merluccius capensis* Cast.) in saturated sodium chloride solution. Other pieces were submerged in a liquid medium consisting of an extract of fish, saturated with sodium chloride. The media were incubated at 30° and 37° C. After several weeks, a red colour developed upon the pieces of fish and also in the purely liquid cultures. Suspensions of red material in saturated salt solution were used to inoculate plates of "fish extract" with 30% sodium chloride and 1.5% agar. The colonies appearing on the plates were all of the same appearance, *viz.*, small, slimy, pink and completely transparent. On no occasion have we encountered different types of colonies and apparently the organism growing and isolated was the only species of strongly halophilic organisms present in our material. It is of importance in connection with older observations (1) that the organism is very resistant in the form in which it is present on the salt. It could still be isolated with ease from a sample of the salt which had been exposed to the very dry laboratory air for more than two years. Direct microscopical examination of slices from the salt showed that the red colour was due to the presence of relatively large red bodies of globular form, floating in a liquid, present in clefts and spaces in the salt crystals.

Attempts to grow the pure strain on peptone agar with 30% salt were unsuccessful and at first the impression was formed that the organism only developed on the "fish extract" medium.

This medium was prepared by boiling 500 g minced stockfish with 1 l tap water for 20 min. The liquid was strained through cloth and filtered. Sodium chloride was then added to a concentration of 30%. Upon sterilisation of this medium, a considerable amount of protein precipitated. At first this precipitate was not removed. The organism developed well on this medium, both at 30° and at 37° C. If, however, the precipitate was removed by filtration, we repeatedly observed that the resultant media only supported growth at the lower temperature and not at 37° C. As a clear medium is more convenient and as growth was excellent at 30° C., we later used the filtered medium exclusively, as it was also more suitable for microscopic examination.

The bacteria remain viable for a very long period on this medium.

A comparison of different kinds of peptone and other nutrients showed that the organism develops abundantly on a solid medium containing in addition to the usual large amount of sodium chloride 1—2 % proteose peptone Difco. The first time there was a considerable lag before growth started, *viz.*, about 18 days. Secondary transfers to the same medium started growing at once. Once the organism was adapted to this kind of nutrient, transfers could also be made to media containing a number of other nutrients. Growth was obtained on sodium chloride media with: Bacto-tryptone, Bacto-tryptose, peptone Witte, beef extract. The amount of growth decreased in this order, on beef extract it was extremely scanty. On all these media the colonies appear opaque in contrast to the translucent colonies obtained on fish extract medium.

The organism is able to liquefy gelatin.

Gelatin media with 30% salt do not set. We poured plates of plain gelatin, which were then impregnated for two days with fish extract 30% sodium chloride. Quantities of bacteria scraped from a slant were put on the plates and liquefaction of the gelatin was observed after two days. There was hardly any growth under these conditions.

In a medium with 28 % sodium chloride and 2 % sodium nitrate, the nitrate was rapidly reduced to nitrite.

An attempt was made to prepare litmus-milk with 30 % salt. This mixture clots, however, during sterilisation. The bacterium develops abundantly on the solid mass, with decolorisation of the litmus.

The organism grows best at salt concentrations from 28 % to saturation. It was found possible to adapt them to a certain extent to lower concentrations. Below 16 % we were, however, unable to obtain growth. It is also possible that there is no real multiplication at lower concentrations, and that the inoculum just remains viable until the salt concentration has become sufficiently high by evaporation. There was a long time lag at such concentrations.

PIGMENTS.

Miss PETTER (2), working with a similar organism, isolated some of the pigments produced by the bacteria and demonstrated that they were carotenoids. We can confirm this observation for our strain. As ours seems to be a strain, poor in pigment production, as compared with some strains, described by other authors, we did not succeed to isolate the pigments in sufficient quantities for a complete characterisation but a few observations may be mentioned here.

The red compounds are not excreted into the medium. The bacteria were collected and extracted with methanol. From this solvent they were transferred to dry hexane and the hexane solution was passed directly over a column of alumina. Development was with a mixture of hexane and benzene 1 : 1, followed by pure benzene. Two zones were obtained in this way, the upper one consisting of a number of non resolved bands. This upper band, after elution with methanol, was chromatographed again in a separate column. This time the solvent was benzene with a trace of ethanol. In total three separate zones were obtained, though there may be more different pigments. The visible absorption spectra of the three zones are recorded in fig. 1. They confirm the conclusion of Miss Petter that the pigments of these bacteria are carotenoids. It is rather certain, however, that in pigment forming properties our strain of bacteria differs from hers.

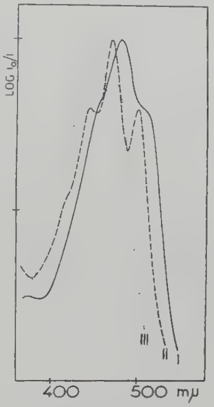


Fig. 1. Absorption spectra of hexane solutions of carotenoids from chromatograms. I: Top zone of second column. II: Bottom zone of second column. III: Bottom zone of first column.

Systematic position.

As to its origin and properties, our organism closely resembles those, described by Klebahn (4), Harrison and Kennedy (1), Petter (2), Volcani (3) and others (5). There can be little doubt that they are all closely related. The chief differences seem to be in the power to reduce nitrates to nitrites, the flagellation and motility, and the proteolytic properties. Like the organisms, described by Petter and Volcani, our isolate reduces nitrate. The organism described by Harrison and Kennedy under the name *Pseudomonas salinaria* lacks this property, as does an apparently very closely related form, described by Lochhead (5).

Whereas Harrison and Kennedy as well as Lochhead reported successful flagella staining with their bacteria, we were unable to demonstrate flagella by staining. Klebahn also failed to stain flagella with his bacterium, which he described under the name of *Bacillus halobius ruber*. Apparently both Petter and Volcani did not find flagella. Most authors who have studied this class of microorganisms agree that ordinary staining is difficult, if not impossible. This would apply even more to flagella stains. It is all a result of the high salt concentration.

We observed on some occasions that a living culture, when examined under the dark field microscope, contains individuals which suddenly become immotile and excrete a thin thread, apparently originating from the contents of the cell, into the medium which coagulates there. Such a happening during attempts to stain the organism may simulate "flagella". In our opinion it is therefore doubtful whether flagella have ever been demonstrated and the question needs further investigation.

A comprehensive study of this unusual group of microorganisms is desirable. For the time being, we would like to follow VOLCANI (l.c.) in placing all rod-like bacteria from salt or salted products, unable to develop in media with salt concentrations below 12—16% NaCl, and producing red carotenoid pigments, together in a genus *Halobacterium*. Sub-divisions could be associated with the power to reduce nitrates, different behaviour towards various protein degradation products, and proteolysis.

STAINING PROPERTIES.

Like most authors, we were not able to stain our organisms directly, but it could be accomplished after fixation with formalin. KLEBAHN obtained satisfactory stains after fixation with iodine.

To fix the bacteria, about 10% by volume of 40% formalin solution is added slowly and with gentle shaking, to a liquid culture of the bacteria. After 3—4 days, the bacteria have settled to the bottom of the vessel. The supernatant is removed for the greater part, and a smear is made from the precipitate in the usual way. Staining is done with carbol fuchsine. Though it is not surprising that this treatment does not leave the bacteria completely undamaged, there are still indications of the particular form (vide infra) of the bacteria in such preparations (fig. 3).

Bacteria treated in this way are Gram negative, same as the deformed amorphous mass, obtained if the bacteria are treated immediately by Gram's method. We have not been able to demonstrate flagella in our formaldehyde-fixed preparations.

MORPHOLOGY, MOTILITY.

Our attention was attracted to these organisms because of their unusual form. Direct microscopic examination of the organisms, suspended in saturated salt solution, did not reveal much detail. A wet preparation from a solid medium, observed with the phase contrast microscope revealed that the majority of individuals did not have the usual cylindrical form of bacteria, but were more or less flat, bent or curled ribbons (fig. 2). To check this observation, use was made of the dark field microscope, with sunlight as a light source (6). This technique proved very suitable for our purpose and it completely confirmed our initial observation. When they are cultured in liquid medium, the organisms usually exhibit a more regular form, the majority being rather straight, flat ribbons. This can be very easily observed because the organisms have a, sometimes rather slow, motion which consists of a rotation around the long axis. One therefore sees the bacteria becoming alternatingly thin and broad depending on whether they are seen on their flat or broad sides.

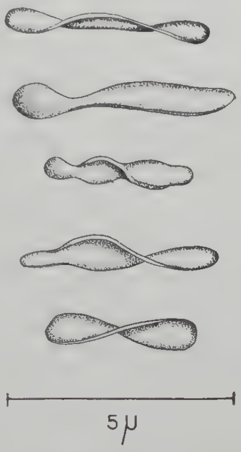


Fig. 2. Forms from agar medium. Phase contrast. Free hand drawing.

A considerable number of individuals were not flat, however, as their bodies showed one or more distinct "twists" (fig. 4). In a non-motile preparation, the interpretation of such forms would of course be very uncertain, but as the bacteria turn round their axis, this twist can be seen "sliding" along the bacterial bodies. In those forms where the two parts were more or less at right angles to one another they were seen alternatingly "en face" so that one part was broad when the other was narrow and vice versa (fig. 41). The photography of these phenomena proved difficult, but we succeeded in obtaining a number of satisfactory pictures. In addition, a 400 ft. motion picture of the bacterium was made. This gives a much better demonstration of the phenomena, observed during the movements of this organism than can be obtained with still pictures.

In the examination of such photographs it should be kept in mind that the eye is much better adapted to evaluate contrasts than the photographic plate. As it is the periphery of the bacterium, the "wall" which scatters the light, the bacteria appear brightest when they show their narrow sides. Consequently, in the photographic picture the bacteria appear thicker than they are in reality or by visual observation.

Because the interpretation of microscopic observations at high power is always somewhat uncertain, we have compared them with observations on glass models. Our microscopic observations were best explained if it is assumed that the light is scattered by the cell wall of the bacterium, the contents of the cell being optically more or less empty. This fits in with present views of the structure of the bacterial cell. This conception will aid in the interpretation of our figure 4.

Our observations leave little doubt that cultures of our bacterium in liquid medium contain a large number of individuals, whose bodies can be described as resembling ribbons, twisted along their long axes to resemble screws. The twist is not always as pronounced as in the cases illustrated. There are also many individuals which appear to be merely cylindrical, but we observed that these are mostly the short, and therefore probably young forms. Forms with more than one twist do occur (fig. 4k and m) but are relatively scarce. In several individuals, the twist is placed so that the part on one side of it is very short. In those cases the shorter part looks like a small knob, joined to the main part by means of a short and very thin "stem" (fig. 4n).

Dark field illumination enabled us to observe another point, not easily noticed by microscopy in transmitted light. Usually, the forward displacement of the bacteria is slow and not very pronounced, so that at first glance, the bacterium may be regarded as non-motile. In the literature there is considerable difference of opinion whether these organisms are motile or not. KLEBAHN described his bacteria as exhibiting a kind of "oscillating" motion, but as he failed to demonstrate flagella, he considered this to be Brownian motion. Realising the flatness of our bacteria and their twist, we saw that this phenomenon is due to real motility and that the organisms rotate around their long axis, sometimes extremely rapidly. The to and fro movement therefore is to be looked upon as a result of this rotation, the whole organism functioning as a ship's screw. Of course, such a kind of motility cannot usually be observed with cylindrical bacteria, but it may well be of wider occurrence than is usually realised. This axial rotation was at times so rapid that it could hardly be followed by the eye. With a not too rapid photographic exposure there may appear a picture formed by the superposition of several consecutive positions of the bacterium. If the bacterium has several twists, such a photograph may resemble a string of beads. Such appearances may have led earlier observers to erroneous conclusions regarding morphology. The reader is referred especially to the photographs on page 45 of Miss

PETTER's thesis (2) which may easily be interpreted in this way. For our photographs we had to choose forms exhibiting relatively slow motion. Individuals can suddenly slow down their rotation, or "accelerate".

In this bacterium we meet with an instance of spiral form in bacteria, though of a somewhat unusual kind. A systematic study of micro-organisms in the living state especially with the aid of dark ground methods might reveal similar phenomena hitherto not noticed because of the inherent limitations of the transmitted light microscope, and fixed preparations.

INCLUSIONS IN THE BACTERIA CAUSING STRONG SCATTERING OF LIGHT.

PETTER (l.c.) noticed in the cells of some of her strains, bodies with an apparently strong refraction. Cultures of such strains had an opaque appearance in contrast to the cultures of other strains. From various experiments she concluded that these bodies were most probably cavities, filled with gas.

We had not observed such inclusions in our strain, until we succeeded in cultivating it on the proteose-peptone medium. Older colonies on this medium showed the opaque appearance reported by PETTER and microscopic examination revealed in the majority of the cells one or more highly scattering particles (fig. 5a and b). These cells were rather cylindrical. There is in our opinion very little support for the idea that the bodies are gas filled cavities. Obviously we have observed here what was described by PETTER. HARRISON and KENNEDY (l.c.) have given a drawing, which suggests that they have seen the same bodies in their bacteria, though they offer a different explanation. Media such as peptones, which were used exclusively by PETTER, are in our experience inferior to the fish extract media and as the bodies develop only when the culture grows old, they had better be looked upon as products of degeneration. Under unfavourable conditions, several bacterial species develop within their cells similar inclusions, which may consist of denatured and thereby condensed protein material.

Summary.

From pink solar salt an obligate halophilic bacterium was isolated and shown to be the cause of the pink discoloration. Method of isolation, cultural characteristics and nature of pigment are de-

tailed. The bacterium proved to be closely related to previously described halophilic bacteria, and VOLCANI's suggestion that all such bacteria should form a genus *Halobacterium* seems acceptable. Further work however is indicated, especially as the present bacterium showed such unusual morphological features. Examined in the live state, by means of phase contrast microscopy, or preferably sunlight dark field methods, this bacterium usually did not present itself as a cylinder, but appeared flat as a ribbon, and in addition this ribbon often showed one or more twists (up to 180°) per individual bacterium. This resulted in a spiral appearance of the bacterium, and its motility seemed to make use of this spiral configuration. Confirmation of this curious kind of morphology and motility was sought and found through the study of spiral glass models, and by means of cine-micrography[1]). Flagella were not found. It is suggested, and there are some indications, that other bacteria of this group, and perhaps of other groups, might on similar examination in the live state, be found to show similar shapes.

Acknowledgements.

Permission was granted to the first author by the Council for Scientific and Industrial Research of the Union of South Africa to publish the results of investigations carried out at the National Chemical Laboratory, Pretoria. Part of the microscopic apparatus used was acquired by the second author through a grant from the same Council. The samples of salt were obtained through the cooperation of Mr. HERMANN OFFEN of Cape Cross, South West Africa.

References.

1. F. C. HARRISON and M. KENNEDY, Trans. Roy. Soc. Canada, Section V, **16**, 101, 1922. – 2. H. F. M. PETTER, Over roode en andere bacteriën van gezouten visch. Thesis Utrecht 1932. – 3. B. ELAZARI-VOLCANI, Studies on the microflora of the dead sea. Thesis Hebrew University, Jerusalem 1940. – 4. H. KLEBAHN, Mitteilungen a.d. Institut f. Allgemeine Botanik (Hamburg) **4**, 11, 1919. – 5. A. G. LOCHHEAD, J. Bact. **27**, 62, 1934; **45**, 575, 1943. – 6. A. PIJPER, J. Path. Bact. **58**, 325, 1946.

---

[1]) Copies of this 16 mm silent film (400 feet) can be obtained at cost price from the second author.

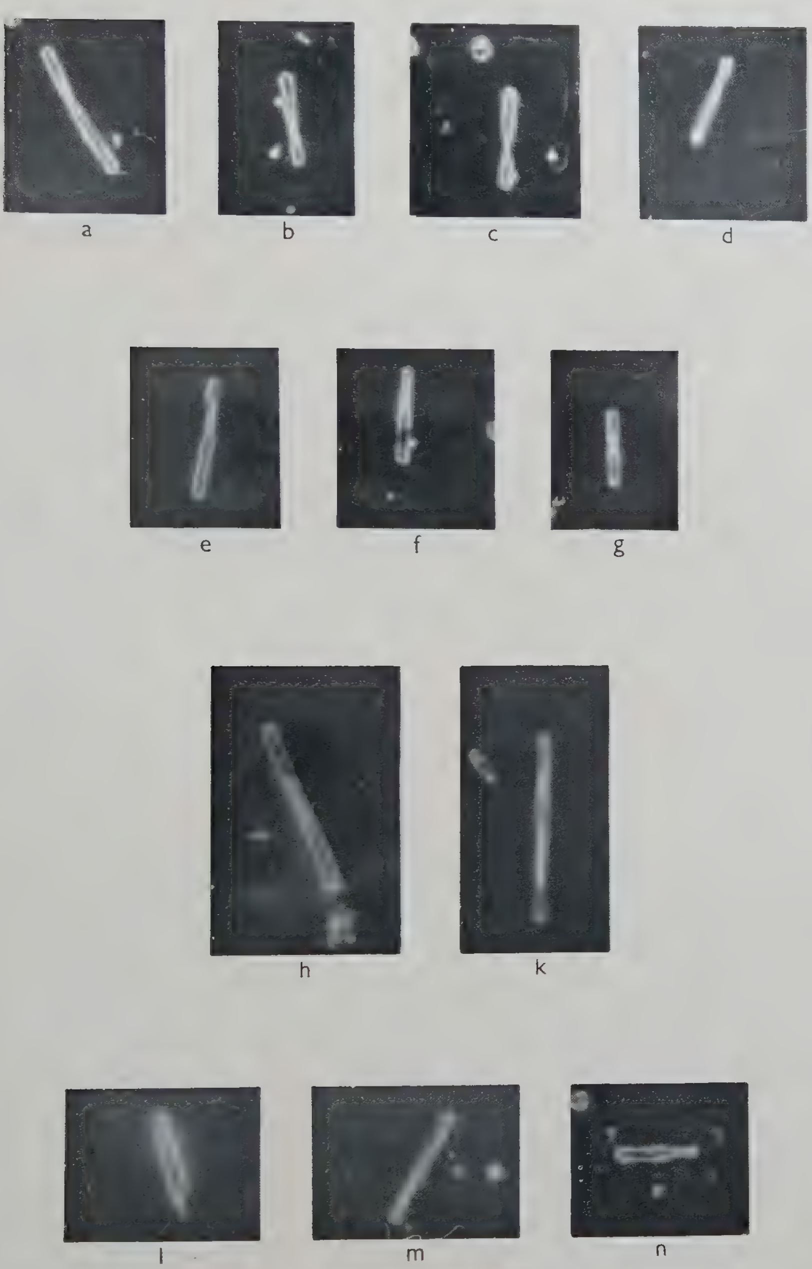


Fig. 4. *Halobacterium* spec. Flat and twisted forms as observed with sunlight dark field illumination. For further reference see text.

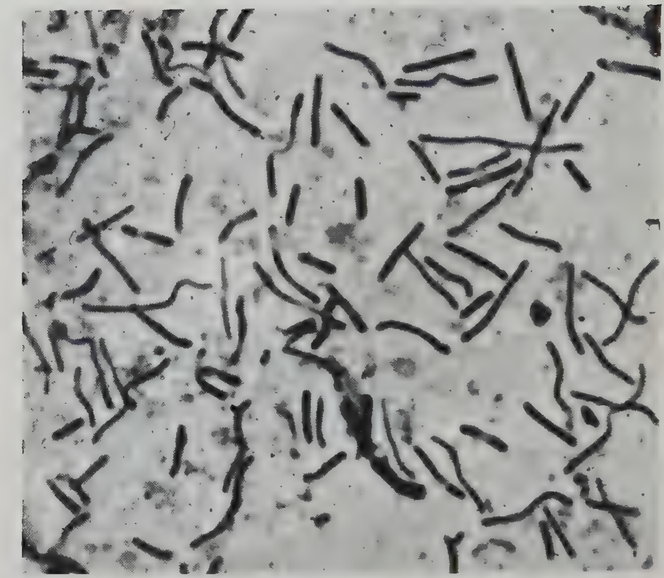


Fig. 3. *Halobacterium* spec. Liquid medium. Fixed with formol. Fuchsine stain. × 1350.

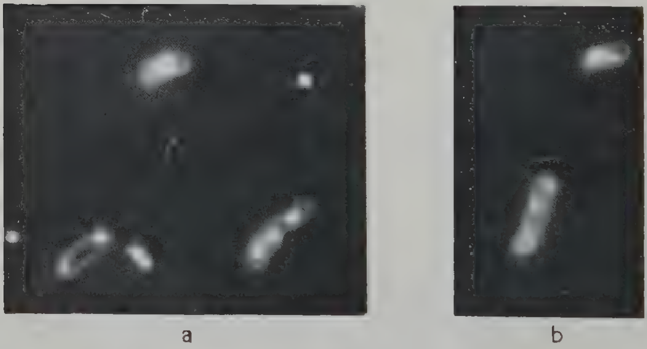


Fig. 5. *Halobacterium* spec. Bacteria from proteose peptone medium, showing inclusions. Sunlight dark field illumination.

(Aus dem Phytopathologischen Laboratorium ,,Willie Commelin Scholten'', Baarn).

UEBER DIE ASCOMYCETENGATTUNGEN *CERATOSTOMELLA* SACC., *OPHIOSTOMA* SYD. UND *ROSTRELLA* ZIMMERMANN

von

J. A. VON ARX

(Eingegangen am 12. Januar 1952).

Im Laufe von Untersuchungen über amerospore Pyrenomycetengattungen wurden wiederum einige Formen gefunden, deren Sporen bei völliger Reife septiert sind. Dazu gehören die typischen Vertreter der Gattung *Ceratostomella*. Da die zahlreichen, teilweise als Erreger von Welkekrankheiten und Blaufäulen besonders für den Forstpathologen wichtigen Vertreter der Gattung *Ophiostoma* auch heute noch öfters mit *Ceratostomella* verwechselt werden, soll vorerst diese Gattung anhand ihrer Typusart etwas eingehender besprochen und abgebildet werden. Anschliessend soll der Formenkreis der Gattung *Ophiostoma* behandelt und ein bisher als *Rostrella coffeae* Zimmerm. bekannter Pilz dazu gestellt werden.

1. *Ceratostomella* Sacc.

Die Gattung *Ceratostomella* wurde von SACCARDO (1878) mit der Typusart *C. vestita* aufgestellt. Wie die Untersuchung des Originalexemplars und einiger anderer Kollektionen ergeben hat, ist dieser Pilz ein Vertreter der Diaporthaceen (sensu MILLER 1949) und ist, wie bereits v. HÖHNEL (1918) erkannt hat, am nächsten mit *Phomatospora* Sacc. verwandt.

Neben *C. vestita* konnten auch mehrere Kollektionen von anderen Arten, vor allem von *C. cirrhosa* nachgeprüft werden. Wie diese Untersuchungen zeigen, lassen sich mehrere der in der Literatur bekannten *Ceratostomella*-Arten nicht aufrecht erhalten, sondern stellen nur Formen einer in ihrem Habitus und ihrer Grösse ziemlich veränderlichen Art dar. Für diese kann der Name *C. cirrhosa* erhalten bleiben. Es ergeben sich folgende Synonyme:

Ceratostomella cirrhosa (Pers.) Sacc. – Syll. fung. **1**, 408, 1882.

Syn.: *Sphaeria cirrhosa* Pers. – Syn. meth. fung. p. 59, 1801.
Ceratostoma cirrhosum Fuck. – Symb. myc. p. 127, 1869.
Ceratostoma albocoronatum Ellis – Americ. Natural. **17**, 318, 1883.
Ceratostomella albocoronata Sacc. – Syll. fung. **9**, 574, 1891.
Sphaeria ampullasca Cke. – Handbook Brit. Fungi no. 2628, 1871.
Ceratostomella ampullasca Sacc. – Syll. fung. **1**, 409, 1882.
Gnomonia de Baryana Auersw. – Mycol. europ. V/VI, p. 23, 1869.
Ceratostomella de Baryana Sacc. – Syll. fung. **1**, 409, 1882.
Ceratostomella capilliformis Bomm. Rouss. Sacc. – Syll. fung. **9**, 573, 1891.
Sphaeria investita Schw. – Syn. Amer. Bor. no. 1621, 1834.
Ceratostomella investita Starb. – Bih. K. Svensk Vet. Ak. Handl. **19**, Afd. 3, no. 2, p. 26, 1894.
Ceratostomella lejocarpa Sacc. – Michelia **1**, 370, 1878.
Ceratostoma multirostratum Fuck. – Symb. myc. p. 129, 1869.
Ceratostomella multirostrata Sacc. – Syll. fung. **1**, 411, 1882.
Sphaeria rostrata Fries – Syst. Myc. **2**, 473, 1823.
Ceratostoma rostratum Fuck. – Symb. myc. p. 127, 1869.
Ceratostomella rostrata Sacc. – Syll. fung. **1**, 408, 1882.
Ceratostomella similis Kirschst. – Krypt. Fl. Brandenb. **7**, 245, 1911.
Ceratostoma subpilosum Fuck. – Symb. myc. p. 128, 1869.
Ceratostomella subpilosa Sacc. – Syll. fung. **1**, 411, 1882.
Ceratostomella Unedonis H. Fabre – Sphaer. Vaucl. **2**, 34, 1883.
Ceratostomella vestita Sacc. – Michelia **1**, 370, 1878.

Der Pilz wächst auf vermorschtem oder verfaulendem Holze. Die Perithecien entwickeln sich dem Substrate eingesenkt, kommen aber oft durch Wegwittern der deckenden Schichten teilweise oder ganz oberflächlich zu stehen. Sie sind schwarz, kugelig oder in der Faserrichtung des Holzes etwas gestreckt, 280—500 μ gross und mit einer am Scheitel oder etwas seitlich stehenden, kurz cylinderischen oder etwas schnabelförmig verlängerten, oft gebogenen, 200—1200 μ langen und 80—150 μ dicken Mündung versehen. Aussen sind sie spärlich oder ziemlich reichlich mit sehr verschieden langen, 3—4 μ dicken, septierten, ziemlich dickwandigen, durchscheinend rotbraunen Hyphen besetzt. Diese können oft völlig fehlen oder sind doch nur spärlich als Nährhyphen an den im Holze sitzenden Teilen der Fruchtkörper zu finden. Oft stehen die Perithecien dicht und sind dann zu zweien oder mehreren verwachsen; diese zusammengesetzten Fruchtkörper bilden dann für jeden Kern eine eigene Mündung. Sehr verschieden geformte und mit sehr verschieden langen Mündungen versehene Perithecien finden sich oft auf demselben Holzstück. Die tiefer im Substrat wachsenden besitzen einen längeren Schnabel als die oberflächlich stehenden; dabei überragt dieser die Substratoberfläche meist deutlich und bildet einen hervorragenden, schwarzen Zapfen (vgl. Fig. 2a).

Die Gehäusewand ist häutig-lederartig und wird höchstens im Alter etwas kohlig-brüchig. Sie besteht aus mehreren, meist 4—7

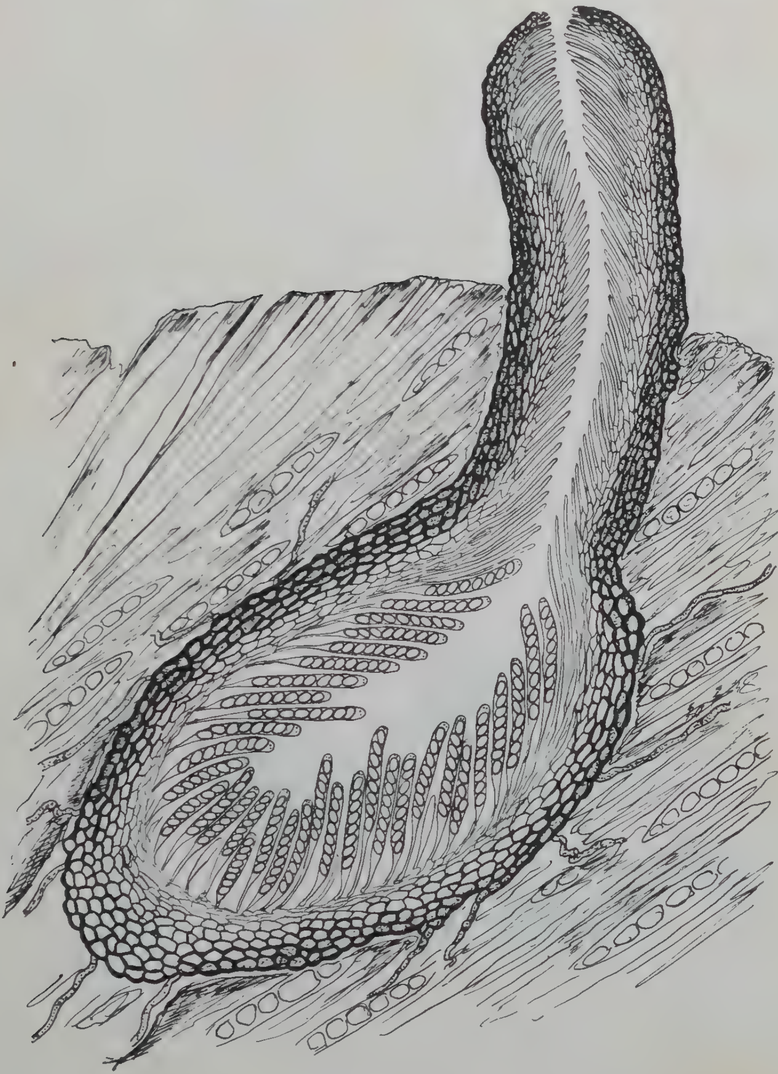


Fig. 1.
Medianschnitt durch einen in der Faserrichtung des Holzes etwas gestreckten Fruchtkörper von *Ceratostomella cirrhosa* (200×).

Lagen von nach aussen dickwandigen, schwarzbraunen, polyedrischen oder etwas zusammengedrückten, in der Mündung gestreckten, 7—16 μ grossen Zellen. Nach innen folgen noch einige Lagen von hyalinen und meist konzentrisch ziemlich stark zusammengedrückten Zellen, so dass die Wand eine Dicke von 25—45 μ erreicht. Die Mündung ist nach oben oft etwas knopfig verdickt; sie ist von einem sehr reich mit dünn- und langfädigen, nach oben gerichteten Periphysen ausgestatteten Porus durchbohrt.

Die Asci entspringen — mehr oder weniger parallel stehend — der ganzen Gehäuseinnenfläche. Sie sind cylinderisch oder etwas keulig, oben oft etwas verjüngt und breit abgerundet, unten in einen verschieden, meist 20—40 μ langen Stiel ausgezogen. Der sporenführende Teil ist 50—80 μ lang und 5—8 μ breit. Die Membran ist dünn und zart; in der Spitze befindet sich ein kleiner, stark lichtbrechender Apikalring. Paraphysen sind meist nur spärlich vorhanden; sie sind dünnfädig und scheinen früh zu verschleimen.

Die acht meist einreihig liegenden Sporen sind länglich, oft

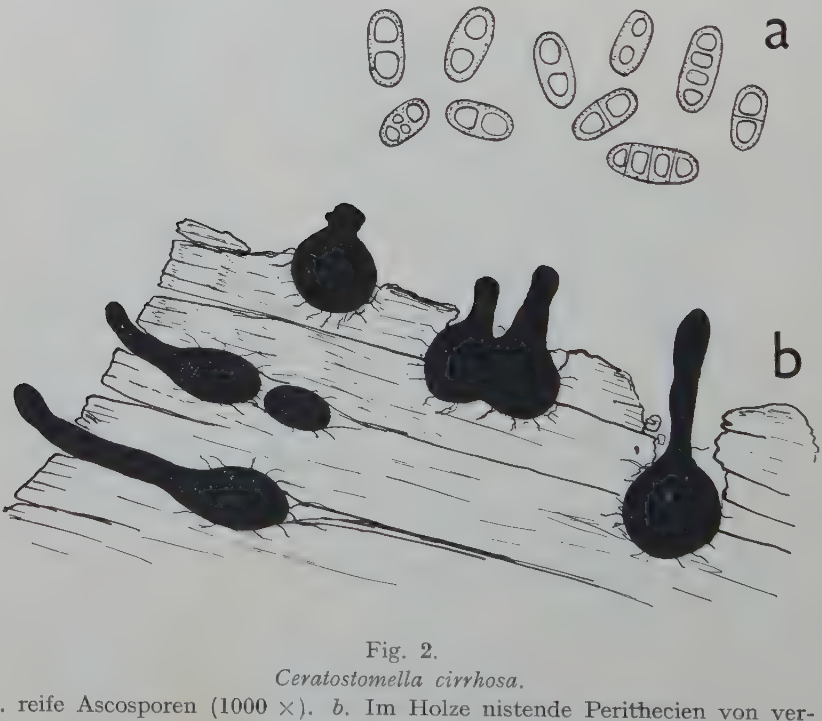


Fig. 2.
Ceratostomella cirrhosa.
a. reife Ascosporen (1000 ×). *b.* Im Holze nistende Perithecien von verschiedener Gestalt (30 ×).

beidends etwas verjüngt und dann breit abgerundet, oft kurz cylinderisch (vgl. Fig. 2a). Erst sind sie hyalin und einzellig, enthalten aber 2 oder 4 grössere Oeltropfen. Reife, sich ausserhalb der Schläuche befindende Sporen sind oft schwach graubräunlich; die Oeltropfen füllen dann das ganze Lumen aus und täuschen manchmal an ihrer Berührungsfläche eine oder drei Querwände vor.

Von diesem Pilz wurden zahlreiche, meist als *C. cirrhosa* bestimmte Kollektionen untersucht. Auch vier aus dem Herbarium PERSOON stammende, als *Sphaeria cirrhosa* bezeichnete ,,Originalexemplare'' konnten nachgeprüft werden. Nur auf einem von diesen konnte der Pilz gefunden werden und auch hier war er nur sehr spärlich vorhanden; die wenigen beobachteten Sporen waren etwa 8—10 μ lang und 3.5 μ breit. Auf zwei anderen Kollektionen konnten nur sehr spärlich leere Gehäuse gefunden werden.

Die Originalkollektion von *C. vestita* Sacc. zeigte den Pilz ebenfalls nur spärlich. Er ist zudem schlecht entwickelt und noch nicht völlig ausgereift; nur innerhalb der Schläuche konnten Sporen gefunden werden. Im Vergleich zu andern Kollektionen waren sie durchschnittlich kleiner. Die Art kann aber von *C. cirrhosa* nicht getrennt werden, da zwischen den beiden Formen alle Uebergänge vorkommen. Wir haben es hier mit einer in ihrer Sporengrösse sehr variablen Art zu tun. Andere, als *C. vestita* bestimmte Kollektionen stimmten dann auch mit der typischen *C. cirrhosa* völlig überein.

Von *C. cirrhosa* standen etwa 30 verschiedene Kollektionen zur Verfügung. Bei einigen konnte der Pilz überhaupt nicht, bei andern konnten nur leere Gehäuse gefunden werden. Kollektionen, die mit einem positiven Ergebnis nachgeprüft und bei denen mindestens je 50 Sporen, nach Möglichkeit ausserhalb der Schläuche, gemessen werden konnten, sind in der folgenden Tabelle zusammengestellt. Dabei sind sie nach der mittleren Sporenlänge geordnet.

Alle hier aufgeführten Kollektionen müssen trotz den abweichenden Sporenmassen in ein und derselben Art untergebracht werden. Die Sporen variieren in ihrer Länge zwischen 5 und 13 und in ihrer Breite zwischen 2.5 und 4.5 μ. In ihrer Form stimmen sie in allen Kollektionen überein; die kleineren enthalten immer nur 2 Oeltropfen, während sich in den grösseren oft 4 befinden.

Neben *C. cirrhosa* in unserem Sinne sind noch einige wahrscheinlich gute Arten beschrieben worden. Diese konnten nicht untersucht werden. Zahlreiche andere Arten aber weichen in ihrem Baue völlig ab und gehören zu *Ophiostoma* Syd.

Bezeichnung	Substrat, Fundort, Sammler, etc.	Sporenmassa
1. *C. vestita*	Fagus: Original Herb. Saccardo	5–6.5 × 2.5–3.5
2. *C. vestita*	Quercus: Belgien, leg. Bommer (Rehm, Asc. 891)	5.5–7.5 × 2.5–3.5
3. *C. vestita*	Holz: Mähr.-Weisskirchen, leg. Petrak 1913	6–9 × 2.5–3.5
4. *C. cirrhosa*	Holz: Uthaca, USA, leg. Atkinson	6.5–10 × 3–4
5. *C. cirrhosa*	Holz: Nassau, Oestrich, leg. Fuckel	7–9 × 2.7–3.7
6. *C. cirrhosa* f. *microspora*	Coniferen: Stockholm, leg. Romell	7–9.5 × 2.5–3.5
7. *C. cirrhosa*	Holz: M.-Weisskirchen, leg. Petrak 1930	7–10.5 × 3.5–4.5
8. *C. vestita*	Holz: leg. Rehm (unreif)	7.5–10 × 3–4
9. *C. cirrhosa*	Pirus malus: Franken, leg. Ade	7–11 × 3–4.5
10. *C. vestita*	Holz: M.-Weisskirchen, leg. Petrak 1913	8–10.5 × 3–4
11. *C. cirrhosa*	Coniferen: San Juan, Wash., USA	8–11 × 2.8–4
12. *C. cirrhosa*	Salix, Mittelfranken (Rehm, Asc. 2020)	8–11 × 3–4
13. *C. cirrhosa*	Fagus: Schweden, Ronneby, leg. Romell	8–11 × 3–4
14. *C. subpilosa*	Acer pseudoplatanus: Deutschl., Kirchstein, leg. Ade	8.5–11 × 3.3–4.3
15. *C. cirrhosa*	Holz: Tirol. Brenner, leg. Rehm	9–12 × 3.5–4.5
16. *C. cirrhosa*	Holz: Finnland, leg. Starbäck	9–12 × 3.5–4.5

2. *Ophiostoma* Sydow und *Rostrella* Zimmermann.

Mit der Diaporthaceengattung *Ceratostomella* stehen nun die Vertreter der Gattung *Ophiostoma* in überhaupt keinem engeren Verwandtschaftsverhältnis. Als Typusart der Gattung hat *Sphaeria pilifera* Fr. zu gelten. Dieser Pilz wurde von WINTER (1887) fälschlicherweise zu *Ceratostomella* gestellt. Dass er nicht dazu gehören kann, erkannte VON HÖHNEL (1918) und begründete für ihn die Gattung *Linostoma*, ohne sich über deren systematische Stellung zu äussern. Da bereits eine ältere Phanerogamengattung *Linostoma* Wallich bestand, haben H. und P. SYDOW (1919) den neuen Namen *Ophiostoma* vorgeschlagen und einige weitere, von HEDGCOCK (1906) und MÜNCH (1908) als *Ceratostomella* beschriebene Arten dazu gestellt.

Die systematische Stellung der Gattung hat NANNFELDT (1932) erkannt. Er begründete ihre Zugehörigkeit zu den Plectascales und stellte sie neben *Microascus* Zukal in die neue Familie der Ophiostomataceen.

Münch (1908) beschrieb einige *Ophiostoma*-Arten mit *Graphium*-Konidien als *Ceratostomella*. Für die übereinstimmend gebaute Ascusform von *Chalara Ungeri* Sacc. stellte er hingegen wegen der abweichenden Konidienform die neue Gattung *Endoconidiophora* mit der Typusart *E. coerulescens* auf. Diese Gattung wurde von Nannfeldt (1934) mit *Ophiostoma* vereinigt. Dieser Autor erwähnt insgesamt 4 Arten mit *Chalara*, *Chalaropsis* oder *Thielaviopsis*-Konidien und sagt, dass die morphologischen Unterschiede zwischen diesen Arten einerseits und den Arten mit z. B. *Graphium*-Konidien anderseits eher kleiner seien als diejenigen innerhalb der Arten der beiden Gruppen.

Wahrscheinlich ohne Nannfeldt's Arbeit zu kennen, hielt Davidson (1935, 1942, 1944) an *Endoconidiophora* neben „*Ceratostomella*" fest und stellte ingesamt fünf weitere Arten dazu. Aber auch dieser Autor kann keine morphologischen Verschiedenheiten im Bau der Ascusform angeben und da sich nach meiner Ueberzeugung auf verschieden gebauten Konidienstadien keine neuen Ascomycetengattungen begründen lassen — schon weil viele dieser Pilze 2 oder 3 verschiedene Konindienstadien in ihren Entwicklungskreis einschliessen, während andere wiederum überhaupt keine Nebenfruchtform besitzen — schliesse ich mich der Ansicht Nannfeldt's an und vereinige hier *Endoconidiophora* mit *Ophiostoma*.

Formen mit Konidien vom Typus der Gattung *Scopularia* Preuss (= *Leptographium* Lagerberg et Melin) hat Goidanich (1936) von *Ophiostoma* abgetrennt und in eine neue Gattung *Grosmannia* gebracht. Diese wurde bereits von Siemaszko (1939) wieder mit *Ophiostoma* vereinigt. Er hat für die von Goidanich zu *Grosmannia* gestellte *O. ips* (Rumb.) Nannfeldt neben der *Scopularia*-Konidienform auch ein *Graphium* nachgewiesen. Mathiesen (1951) erwähnt ebenfalls eine Form die Charakteristiken von *Ophiostoma* und *Grosmannia* in sich vereinigt. Ihrer Ansicht, dass auch diese Gattung — wie *Endoconidiophora* — als Sektion zu *Ophiostoma* zu stellen ist, pflichte ich bei.

Zu *Ophiostoma* muss auch der einzige Vertreter der Gattung *Rostrella* gebracht werden. Diese wurde von Zimmermann (1900) für den Erreger einer auf *Coffea arabica* auftretenden Welkekrankheit aufgestellt. Der Name *Rostrella* ist älter als *Ophiostoma* (Sydow 1919) und hätte daher die Priorität; er muss aber verworfen werden, da er bereits von Fabre (1878) für einen ganz anders gebauten, mit *Lophiostoma* verwandten Ascomyceten gebraucht wurde.

Vor einiger Zeit erhielten wir aus Paramaribo (Suriname) eine Sendung abgestorbener Aeste des Kaffeebaumes. Auf diesen wurden reichlich Perithecien eines Pilzes gefunden, die sicher mit *R. coffeae* identisch sind. Da die Publikation von ZIMMERMANN in holländischer Sprache verfasst wurde und vielerorts wahrscheinlich nicht leicht erhältlich ist, soll die Art hier etwas ausführlicher beschrieben und abgebildet werden.

Die Perithecien wachsen oberflächlich auf Holz oder Rinde in grösseren, lockeren oder dichten Herden oder stehen zu kleinen

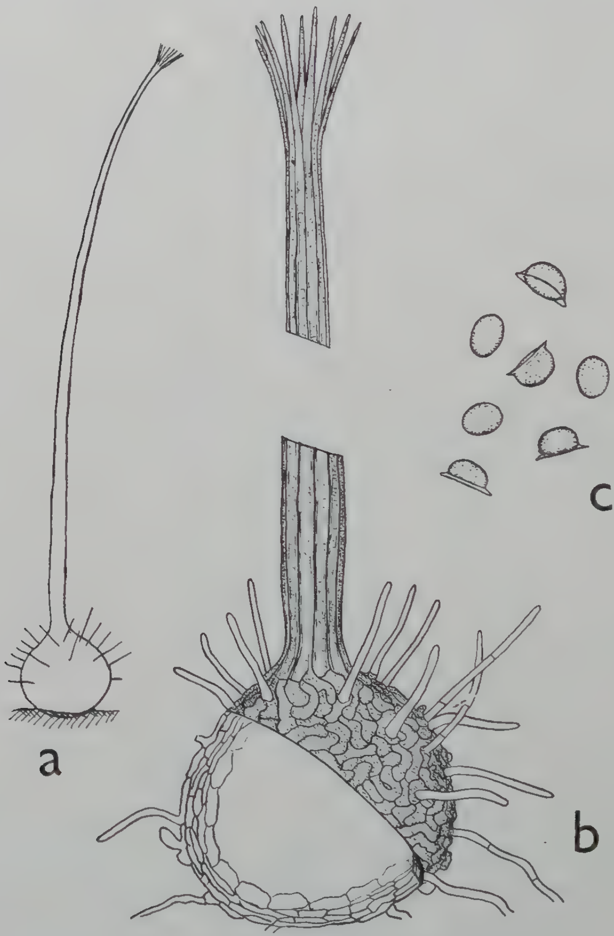


Fig. 3.
Ophiostoma coffeae. a. einzelnes Perithecium, Uebersichtsbild (50 ×); *b.* Perithecium mit Mündung (350 ×); *c.* Ascosporen (1000 ×).

Grüppchen vereinigt. Sie sind kugelig, schwarz, 100—160 μ gross und sind mit einem langen Mündungsschnabel versehen. Dieser ist pfriemenförmig, meist gerade, 500—1000 μ lang, unten 22—30, oben 14—20 μ dick. Die Gehäusewand ist dünn und besteht aus miteinander verflochtenen Hyphen, die aus sehr unregelmässigen, meist langgestreckten und gewundenen, hellbraunen, zelligen Gliedern aufgebaut sind. Im Schnabel ordnen sich diese zu parallelen Reihen, sind sehr langgestreckt und laufen an der Spitze zu 8—13 starren, subhyalinen, zugespitzten, 15—70 (meist 40—50) μ langen Wimperhaaren aus. Unten ist der Schnabel ziemlich dunkel olivenbraun, wird nach oben heller und ist in der Spitze fast subhyalin. In seltenen Fällen sind die Wimperhaare sehr kurz oder fehlen völlig. Die Perithecien sind kahl oder häufiger mit olivenbraunen oder nach ihren Enden zu fast subhyalinen, 3—8 μ dicken und 30—70 μ langen Hyphen besetzt.

Die sehr vergänglichen, kugeligen oder ellipsoidischen Asci füllen das ganze Fruchtkörperinnere aus. Die Sporen sind von oben gesehen breit ellipsoidisch, von der Seite mehr oder weniger halbkugelig oder helmförmig und sind mit einem ringförmigen, manchettenartigen Häutchen versehen. Sie messen 4,5—6 × 3 — 4 μ, gelangen durch den Schnabel ins Freie und sammeln sich im Wimperkranze in einem dichten, schleimigen Tröpfchen.

Der Pilz besitzt insgesamt 3 verschiedene Konidienformen, die aber teilweise nur spärlich gefunden werden konnten. Reichlich fanden sich Chlamydosporen; diese entstehen terminal an kurzen, oft etwas verzweigten und bald verschrumpfenden Trägern. Sie sind kugelig oder birnförmig, erst hyalin, später braun, mit glattem, ca. 1 μ dickem Epispor, 9—18 μ gross.

Ferner gehören zu Pilze zwei Endokonidienformen. Bei der einen entstehen in unten bräunlichen, nach oben subhyalin werdenden und sich etwas verbreiterden, büchsenförmigen Trägerhyphen rundlich-würfelförmige oder kurz zylindrische, an den Enden dann breit abgerundete, 8—16 μ lange und 6—9 μ breite, hyaline, einzellige, in oft langen, perlschnurartigen Ketten stehende Konidien. Bei der andern Form sind die Konidienträger 80—160 μ lang, unten oft etwas bauchig, braun, mit 2 oder 3 Querwänden, nach oben flaschenhalsförmig verschmälert. In ihnen entstehen stäbchen- oder cylinderförmige, einzellige, hyaline, 10—25 μ lange und 3—5 μ breite Sporen.

In Kultur auf Glucose-Kartoffel-Agar beträgt der tägliche Zuwachs des Mycels 0,5 cm bei 22° C.; 0,8 cm bei 30° C. Der Pilz bildet ein spärliches oder oft ziemlich dichtes, schmutzigweisses Luftmycel. Die im Agar wachsenden Hyphen sind vorerst ebenfalls hyalin, färben sich aber bald bräunlich oder rotorange, so dass die Kultur mit zunehmendem Alter ein dunkelbraunes Aussehen erhält. Die im Agar wachsenden Hyphen sind reich septiert und 4—7 μ dick, während die Hyphen des Luftmycels nur 2—5 μ dick werden. An ihnen entwickeln sich meist lange und dünne, oft quirlig stehende Trägerhyphen, an denen endogen oder exogen (nach dem Phialophora-Typus) sehr zahlreich hyaline, einzellige, 5,5—13 μ lange und 2,5—5 μ breite Konidien entstehen. Oft zerfallen auch längere Mycelstücke Oospora-artig in zahlreiche, einzellige Konidien. In älteren Kulturen finden sich ferner interkalar und terminal zahlreiche Chlamydosporen.

Dieser Pilz hat nun *Ophiostoma coffeae* (Zimmerm.) comb. nov. (syn.: *Rostrella coffeae* Zimmerm.) zu heissen. Er ist ein typischer Vertreter der Sektion *Endoconidiophora.* ZIMMERMANN (1900) hatte ihn sehr ausführlich beschrieben und abgebildet. Er hatte auch seine systematische Stellung ganz richtig erkannt, was aus den folgenden Ausführungen hervorgeht (übersetzt): ,,Der Einteilung der Ascomyceten in den Natürl. Pflanzenfamilien von ENGLER und PRANTL (Teil 1, Abt. 1) folgend, gehört *Rostrella coffeae* zu den Plectascineen in die Familie der Aspergillaceen. Sie scheint am besten mit *Microascus* Zukal übereinzustimmen, weicht aber von dieser Gattung doch so stark ab, dass die Bildung einer neuen Gattung berechtigt erscheint.''

PONTIS (1951) hat den Kaffeekanker ebenfalls in Südamerika (Columbia) auf *Coffea arabica* L. beobachtet und glaubt *Rostrella coffeae* mit *Ophiostoma fimbriata* (Ellis et Halst.) Nannf. (syn.: *Ceratostomella fimbriata* Elliot, *Endoconidiophora fimbriata* Davidson) identifizieren zu müssen. Diese Art ist aber nach den von HARTER und WEIMER (1929) und von ANDRUS und HARTER (1933) gegebenen Beschreibungen verschieden, zum Beispiel fehlt bei den rundlichen Ascosporen das manchettenartige Häutchen. Dieser Pilz verursacht auf *Ipomoea batatas* (L.) Lam. eine als ,,black rot'' bezeichnete Krankheit, und nach PONTIS (1951) vermag er den Kaffeebaum nicht anzutasten.

C. et M. MOREAU (1951) erwähnen bei der Bespechung der von ihnen neu beschriebenen *Gnomoniella quadriguttulata* auch *Rostrella* *coffeae* und sagen, dieser Pilz sei von DADE (1928) zu *Ceratostomella paradoxa* [= *O. paradoxa* (Dade) Nannf. und nicht *O. paradoxa* (de Seynes) Dade] gebracht worden. In Wirklichkeit aber erwähnt DADE die *Rostrella* mit keinem Wort und diese ist auch von *O. paradoxa* deutlich verschieden. Letztere hat viel grössere und dickwandigere, parenchymatisch aufgebaute Perithecien und bei den Ascosporen fehlt das für *O. coffeae* typische, ringförmige Häutchen.

Gnomoniella quadriguttulata C. et M. Moreau kann übrigens, wie aus der guten Abbildung und der ausführlichen Diagnose hervorgeht, nicht bei *Gnomoniella* bleiben. Diese Gattung besitzt spindelförmige, sehr dünnwandige, langgestielte und sich früh loslösende, auf verschiedener Höhe stehende Asci (vergl. KLEBAHN 1908). *G. quadriguttulata* entspricht aber sehr gut der Gattung *Ceratostomella* und ist von *C. cirrhosa* kaum verschieden.

Einige weitere, in den letzten Jahren als *Ceratostomella*, *Endoconidiophora* oder *Grosmannia* beschriebene Arten müssen ebenfalls umbenannt werden. Es sind dies:

1. *O. multiannulatum* (Hedgc. et Davidson) comb. nov.
Syn.: *Ceratostomella multiannulata* Hedgc. et Dav. – Journ. agr. Res. **50**, 797, 1935.
2. *O. obscurum* (Davidson) comb. nov.
Syn.: *Ceratostomella obscura* Davidson – Journ. agr. Res. **50**, 798, 1935.
3. *O. piceaperdum* (Rumbold) comb. nov.
Syn.: *Ceratostomella piceaperda* Rumbold – Journ. agr. Res. **52**, 432, 1936.
4. *O. pseudotsugae* (Rumbold) comb. nov.
Syn.: *Ceratostomella pseudotsugae* Rumbold – Journ. agr. Res. **52**, 426, 1936.
5. *O. lunae* (Kitajima) comb. nov.
Syn.: *Endoconidiophora lunae* Kitajima – Bull. imp. For. Exp. Stat. **35**, 126, 1936.
Matr.: *Fagus crenata* (Japan).
6. *O. serpens* (Goidanich) comb. nov.
Syn.: *Grosmannia serpens* Goid. – Boll. R. Staz. Pat. Veg. Roma **16**, 1936.
7. *O. montium* (Rumbold) comb. nov.
Syn.: *Ceratostomella montium* Rumbold – Journ. agr. Res. **62**, 597, 1941.
8. *O. radicicolum* (Bliss) comb. nov.
Syn.: *Ceratostomella radicicola* Bliss – Mycologia **33**, 468, 1941.
9. *O. leptographoides* (Davidson) comb. nov.
Syn.: *Ceratostomella* (*Grosmannia*) *leptographoides* Davidson – Mycol. **34**, 657, 1942.
10. *O. microsporum* (Davidson) comb. nov.
Syn.: *Ceratostomella* (*Ophiostoma*) *microspora* Dav. – Mycol. **34**, 650, 1942.

11. *O. rostrocylindricum* (Davidson) comb. nov.
Syn.: *Ceratostomella (Ophiostoma) rostrocylindrica* Davidson – Mycol. **34**, 658, 1942.
12. *O. virescens* (Davidson) comb. nov.
Syn.: *Endoconidiophora virescens* Davidson – Mycol. **36**, 301, 1944.
13. *O. variosporum* (Davidson) comb. nov.
Syn.: *Endoconidiophora variospora* Davidson – Mycol. **36**, 303. 1944.

Die Gattung *Ophiostoma*, wie sie von NANNFELDT (1934) charakterisiert und aufgefasst wurde, stellt sicher eine natürliche Einheit dar. Schon wegen ihrem grossen Umfang wäre es wünschenswert, sie in natürliche, gut charakterisierte Sektionen unterteilen zu können. Dies kann aber nur im Rahmen einer monographischen Bearbeitung des gesamten Formenkreises unter Einbeziehung von möglichst allen Arten erfolgen.

Für die Gattung ergibt sich nun folgende Synonymie:

Ophiostoma Sydow — Ann. Myc. **17**, 43, 1919 prop. nom. cons. Nannfeldt 1934.

Syn.: *Rostrella* Zimmerm. – Meded. 's Lands Plantentuin **37**, 24, 1900; non FABRE 1878.
Endoconidiophora Münch – Naturw. Ztschr. Forst und Landw. **6**, 34, 1908.
Linostoma v. Höhnel – Ann. Myc. **16**, 91, 1918; non WALLICH.
Grosmannia Goidanich – Boll. R. Staz. Pat. veg. Roma **16**, 26, 1936.
Ceratostomella auct. non SACCARDO (1878).

Die untersuchten *Ophiostoma*-Kulturen stammten aus dem Centraalbureau voor Schimmelcultures in Baarn, dort wurde auch eine Kultur von *Ophiostoma coffeae* deponiert. Das untersuchte Herbarmaterial von *Ceratostomella* stammte aus dem Naturhistoriska Riksmuseet in Stockholm, dem Rijksherbarium in Leiden und dem Istituto Orto Botanico in Padua. Den genannten Institutionen sei auch hier für die Bereitwilligkeit, mit der sie mir die betreffenden Pilze zur Verfügung stellten, gedankt.

Summary.

Ceratostomella Sacc. is a member of the *Diaporthaceae* (sensu MILLER 1949) and is closely related to *Phomatospora* Sacc., if *Ceratostomella vestita* Sacc. [= *C. cirrhosa* (Pers.) Sacc.] is regarded as type of the genus. This species has perithecia with a rather thick membrane, consisting of several layers of slightly compressed, dark cells. The neck is long cylindrical, lined with periphyses. The asci

are cylindrical, with an refractive ring in the thickened tip. When young, the spores are continuous, hyaline, with two or four oildrops, when old they often develop one or three pseudosepta. A list of synonyms of this species is given.

Some species, which have been described as *Ceratostomella* belong to *Ophiostoma* Sydow (1919); this genus belongs to the *Plectascales* (NANNFELDT 1932, 1934). *Rostrella coffeae* Zimmerm. is also an *Ophiostoma* and must be named *O. coffeae* (Zimmerm.) comb. nov. The name *Rostrella* Zimmermann (1900) is older than *Ophiostoma*, but must be rejected, as an ascomycetous genus *Rostrella* Fabre exists, described in 1878. Several other species, described as *Ceratostomella, Endoconidiophora* and *Grosmannia* are transferred to *Ophiostoma.* A list of synonyms of the genus is given.

Literatur.

1. R. E. ANDRUS and S. L. HARTER, Journ. agr. Res. **46**, 1059, 1933. – 2. H. A. DADE, Transact. Brit. Myc. Soc. **13**, 184, 1928. – 3. R. W. DAVIDSON, Journ. agr. Res. **50**, 789, 1935. – 4. R. W. DAVIDSON, Mycologia **34**, 650, 1942. – 5. R. W. DAVIDSON, Mycologia **36**, 300, 1944. – 6. J. H. FABRE, Ann. sc. Nat. sér. 6, Botanique **9**, 66, 1878. – 7. G. GOIDANICH, Boll. R. Staz. Pat. Veg. Roma **16**, 26, 1936. – 8. L. L. HARTER and J. L. WEIMER, U.S. Dept. Agr., Techn. Bull. **99**, 1929. – 9. C. G. HEDGCOCK, Missouri Bot. Gard., Ann. Rep. **17**, 1906. – 10. F. v. HÖHNEL, Ann. Myc. **16**, 91, 1918. – 11. H. KLEBAHN, Ztschr. f. Pflanzenkrankh. **18**, 129, 1908. – 12. A. MATHIESEN, Svensk Bot. Tidskr. **45**, 203, 1951. – 13. J. H. MILLER, Mycologia **41**, 99, 1949. – 14. C. et M. MOREAU, Revue de Mycologie, Suppl. Colonial **16**, 12, 1951. – 15. J. A. NANNFELDT, Nov. Act. Reg. Soc. Sci. Ups. sér. 4, **8**, no.2, 1932. – 16. J. A. NANNFELDT in: E. MELIN and J. A. NANNFELDT, Svensk Skogsvårdsfören Tidskr. **3/4**, 1934. – 17. R. E. PONTIS, Phytopathology **41**, 178, 1951. – 18. P. A. SACCARDO, Michelia **1**, 370, 1878. – 19. W. SIEMASZKO, Planta Polonica **7**, 1, 1939. – 20. H. und P. SYDOW, Ann. Myc. **17**, 43, 1919. – 21. G. WINTER, Ascomyceten II in RABENHORST's Kryptogamenflora 1887. – 22. A. ZIMMERMANN, Meded. uit 's Lands Plantentuin **37**, 24, 1900; Bull. de l'institut botanique de Buitenzorg **4**, 19, 1900.

(Laboratory of Bacteriology and Hygiene of Domestic Animals,
State University of Ghent).

STUDIES OF THE ANTIGENIC PROPERTIES OF FOWL ERYTHROCYTES

by

L. M. G. GEURDEN and A. DEVOS

(Received February 25, 1952).

INTRODUCTION.

The hemagglutination of fowl erythrocytes by means of viruses (HIRST (3)) may be considered as the starting point of a series of methods for identification of a group of viruses among which: hemagglutination inhibition test (LUSH (4)), sensito-hemagglutination (BANG and LIBERT (1)), etc. All these investigations were chiefly carried out with the virus of Newcastle disease. We as well used this virus for the above mentioned reactions.

The hemagglutination test is not reversible, because the virus is adsorbed on the erythrocytes and the sensito-hemagglutination cannot be obtained, when this adsorption has not previously been realized. It was our aim to ascertain in which state the virus is adsorbed on the erythrocytes; after inoculation of sensitized fowl red cells in chick embryos the virus cannot be separated from the erythrocytes and culture of the virus in the allantoic fluid cannot be obtained. For this reason we have tried by means of hemolysis of normal and sensitized erythrocytes to study separately the properties of the cell walls (stromas) and the hemolysate.

It then appeared:

1. that after hemolysis normal cell walls may be agglutinated by NCD virus in the same manner as complete erythrocytes,

2. that sensitized erythrocytes after hemolysis leave behind sensitized cell walls that may be agglutinated by normal fowl erythrocytes and by blood serum containing sensito-hemagglutinins,

3. that sensitation may be realized on normal cell walls after hemolysis of normal fowl red cells.

All these findings demonstrate clearly that the properties showed in the hemagglutination by complete fowl erythrocytes are exclusively situated in the cell walls of these erythrocytes. These results have led us to ascertain the antigenic properties of the different elements of fowl red cells.

ANTIGENS OF FOWL ERYTHROCYTES.

For this investigation we have taken 4 batches of two rabbits each in which we have injected:

1. suspensions of washed fowl erythrocytes,
2. suspensions of cell walls of washed fowl erythrocytes,
3. hemolysate of normal fowl red cells,
4. normal blood serum of chickens.

Obviously in the batches 1 and 4 we must obtain: in 1 an agglutinating, resp. hemolysing antiserum and in 4 a precipitating antiserum. We actually obtained in 1 a hemolysing and hemagglutinating serum which gave a positive reaction until a dilution 1/800. As to 4 we obtained a precipitating serum giving a positive reaction until a dilution 1/2000. With the serum of 2 we were not able to make a slow agglutination test, because the suspensions of fresh cell walls are not stable for a longer period; nevertheless the rapid agglutination was clearly positive. With a suspension of normal fowl erythrocytes and antiserum 2 we executed a slow agglutination test; the reaction was positive until a dilution 1/50 and by a rapid test using the serum 1 and a suspension of normal cell walls the reaction was strongly positive.

The precipitation test with hemolysate of normal fowl red cells used as an antigen together with serum 3 was negative, but probably this is to be attributed to an insufficient concentration of the hemolysate. Serum 3, however, brought into contact with normal fowl serum gave a positive precipitation until a dilution 1/20. We did not intend to look for specific precipitation reactions with the different protein fractions present in normal blood serum of chickens such as been done by BOYDEN *et al.* (2). In our opinion, however, the positive reaction obtained with serum 3 against normal fowl serum proves that the support of hemoglobin may be a protein fraction identic with one of those present in the blood serum.

As to the hemagglutination reactions our findings show that here only the cell walls play a role.

CONSERVATION OF ANTIGENIC PROPERTIES OF CELL WALLS AFTER LYOPHILIZATION.

In our opinion it was of importance to ascertain whether the above mentioned properties of fowl red cells, which could be localized in the cell walls, would be preserved after lyophilization. Fresh suspensions of fowl erythrocytes can stand only a short storage in the refrigator; on the contrary lyophilized cell walls stand storage in closed vials for a long period even at room temperature.

The results of experiments carried out by us show that all the properties of fresh cell walls may be found back in lyophilized cell walls. Nevertheless stabilisation of suspensions of lyophilized cell walls might become possible after shaking the latter in saline by means of a vibro-motor. The opportunity, however, to verify this assertion was lacking.

Summary.

While in all the hemagglutination reactions with viruses the properties showed by complete fowl erythrocytes are exclusively situated in the cell walls, the authors have studied the antigenic properties of both elements of fowl red cells obtained after hemolysis. Cell walls provoke in the blood serum of rabbits formation of hemolysin and agglutinin just as entire normal red cells do. Probably hemolysate contains a protein fraction identic with one in the blood serum. All the antigenic properties of fresh cell walls are preserved after lyophilization.

References.

1. F. BANG and R. LIBERT, Bull. J. Hopkins Hosp. **85,** 416, 1949. – 2. A. BOYDEN, R. DEFALCO and D. GEMEROY, The Serological Museum Bull. **6,** 6, 1951. – 3. G. HIRST, Science **94,** 22, 1941. – 4. D. LUSH, Journ. Comparat. Patholog. **53,** 157, 1943. – 5. L. GEURDEN, Rev. Belge de Path. et de Med. exp. **21,** 169, 1951.

(Laboratory of Hygiene, University of Utrecht, Netherlands).

THE TUBERCULOSTATIC ACTION OF VITAMIN D_2 [1]

by

W. KLIP

(Received March 2, 1952).

The influence of vitamin D_2 and related steroids in several forms of tuberculosis has been investigated by many authors since 1925, when MALMSTRÖM (13) first conceived the idea of this therapy. Early reports as to the value of this treatment differ, but during the second world war the favourable influence on lupus vulgaris of a daily dose of ± 4 mg vitamin D_2 (calciferol) orally was irrefutably proved by extensive investigations of FANIELLE in Belgium, DOWLING and PROSSER THOMAS (6) in England and CHARPY (2) in France.

Vitamin D_3 or intramuscular injection of high doses of ergosterol have analogous results (GROEN (9)).

As to the mechanism of this therapy opinions differ. RAAB (16) states that calciferol in a concentration of 50 units (1.25 μ g) per ml medium (Hohn, Loewenstein, potato medium) inhibits the growth of virulent tubercle bacteria and has a direct bactericidal, bacteriostatic and bacteriolytic action in vitro and in vivo. BRETT and ECKES (1) could not confirm these results nor could WALKER (18). He reported that tubercle bacteria incubated in serum containing 500 I.U./ml calciferol for 24 hours retained a normal morphology and virulence. On Dorset's egg medium, WETHERLEY-MEIN (17) obtained normal growth in the presence of 10.000 I.U. calciferol per ml.

PICHAT, VIALLIER and MINJAT (15) found a slight inhibitory action of high concentrations vitamin D_2 (600 μ g/ml) in submerged cultures of avirulent *M. tuberculosis*. They report further that serum, urine and pleural exsudate of patients treated with calciferol do not inhibit growth.

---

[1] This work was completed with finantial aid of the National Health Research Council (T.N.O.), the Hague, Netherlands.

In animal experiments, positive results were only reported by RAAB (16) and MEERSEMAN (14). According to GIROUX (8) the resistance of guinea-pigs against infection with *M. tuberculosis* is strengthened by previous administration of calciferol, but after infection the administration of calciferol was without influence. Negative results were further reported by LEVADITI and LI JUAN PO (12), DICKINSON (5) and for vitamin D_3 by JOSSERAND, PICHAT, VIALLIER and KALB (10).

The therapeutic value of calciferol in tuberculosis was ascribed to *a*) promotion of calcification of lesions, *b*) activation of tissue phosphatases and acidosis (CHARPY (2)), *c*) remineralisation of the cutis (BRETT and ECKES (1)), and *d*) antihistaminic activity of calciferol (DAINOW (4)). The author supposes (KLIP (11)) that calciferol interferes with the phospholipid turnover of *M. tuberculosis* and of the granulation tissue. The interference with tissue metabolism is an unfavourable component counteracting the action against the bacteria.

This paper reports on the influence of calciferol, vitamin D_3, cholesterol and ergosterol on the growth of virulent and avirulent strains of *Mycobacterium tuberculosis* in vitro.

MATERIALS AND METHODS.

Twelve strains of *M. tuberculosis* were used: an avirulent human strain growing homogeneously (strain D_{328}), an avirulent bovine strain (B.C.G.) only growing on the surface of the medium and ten virulent human strains with surface growth. Strain A_{30} was isolated from sputum, strains I unto IX were isolated from lupus tissue [1]).

Sauton's medium was used throughout; in some experiments 0.5% serum albumin was added. The substances under study were dissolved in ethanol. It was shown that small concentrations of ethanol in the medium (never exceeding 1.5%) did not affect growth.

Strain D_{328} was cultivated in glass tubes (220 × 25 mm) containing 20 ml medium and adapted for direct use in an extinctometer. A standard curve relating turbidity to bacterial weight was prepared and growth expressed as bacterial weight per ml. Strain A_{30} was cultivated on the surface of 25 ml of Sauton medium in 50 ml flasks.

---

[1]) I am greatly indebted to Dr VAN DER HOEDEN, Tel Aviv, who isolated the strains I—IX.

Surface growth was expressed as the quotient area of growth/area of inoculum. Areae were measured by comparison with paper circles with known radii. The error of this procedure is satisfactory. Fourty readings of the same membrane (and the inoculum) gave fourty quotients among which only one differed more than 25%

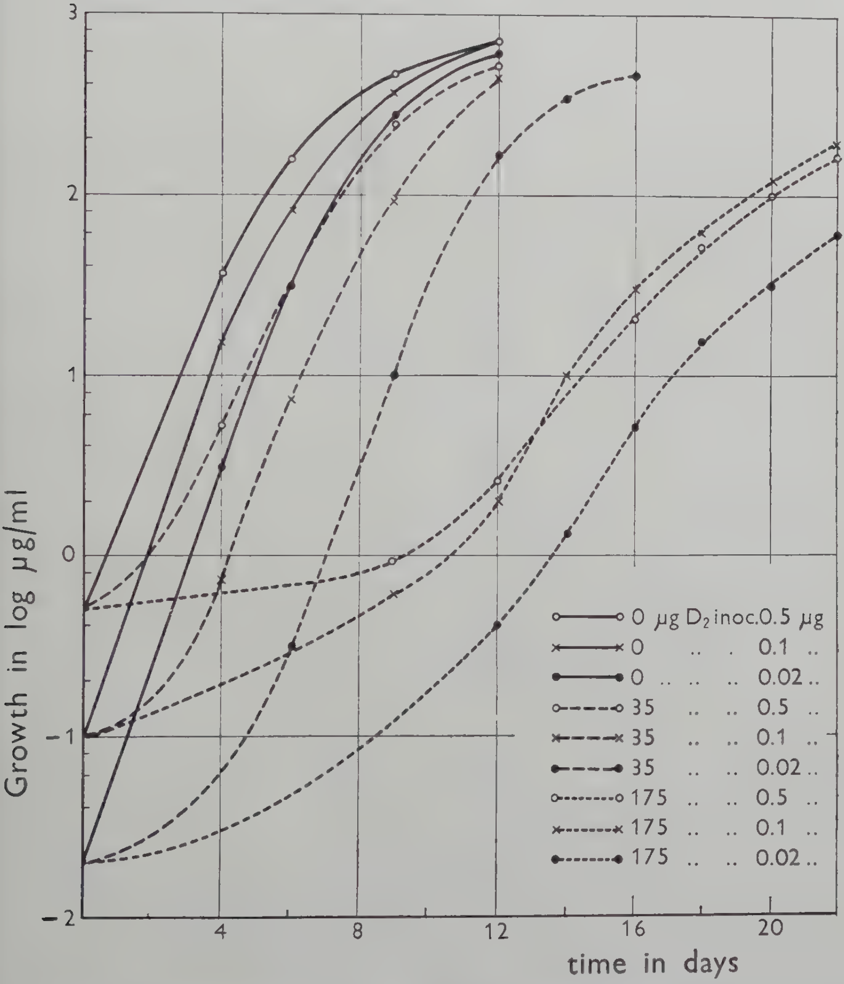


Fig. 1.

Influence of calciferol concentration and size of inoculum on growth of *M. tuberculosis* (strain D_{328}) in Sauton.

from the mean. Each experiment was done in quintuple and each point in the graphs is the mean from the observations in the 5 similar flasks of the experiment.

RESULTS.

Growth inhibiting influence of calciferol on *M. tuberculosis* D_{328}.

Various dilutions of calciferol in Sauton's medium were inoculated with known quantities of a culture of D_{328} in Sauton

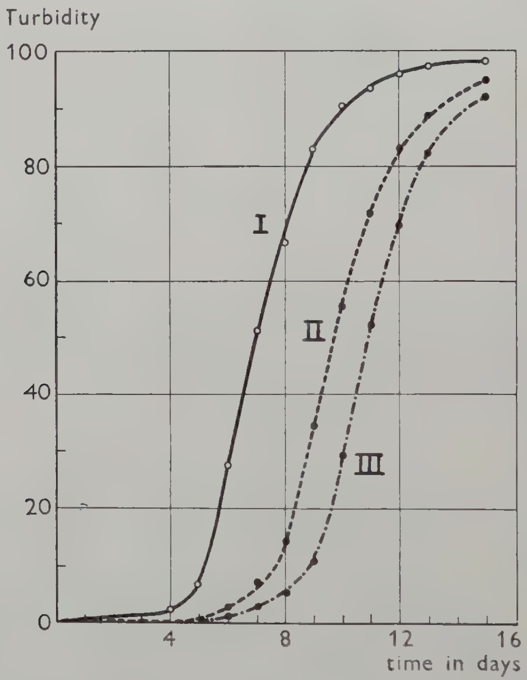


Fig. 2.

Influence of destruction products of calciferol on growth of *M. tuberculosis* (D_{328}) in Sauton.

I. controls.
II. 35 μ g calciferol added seven days before inoculation.
III. 35 μ g calciferol added immediately before inoculation (no calciferol could be found by chemical means in medium II at the time of inoculation).

and incubated at 37° C. All series were prepared in triplicate. The turbidity was measured at regular intervals. Results are represented in fig. 1 (see p. 219).

A concentration of 35 µg/ml calciferol increases the apparent lag time with 1 to 4 days, dependent on the inoculum size. Higher concentrations calciferol also affect the slope of the growth curve and the final concentration of bacteria. With very high concentrations (400 µg/ml) no growth was obtained (not shown in fig. 1).

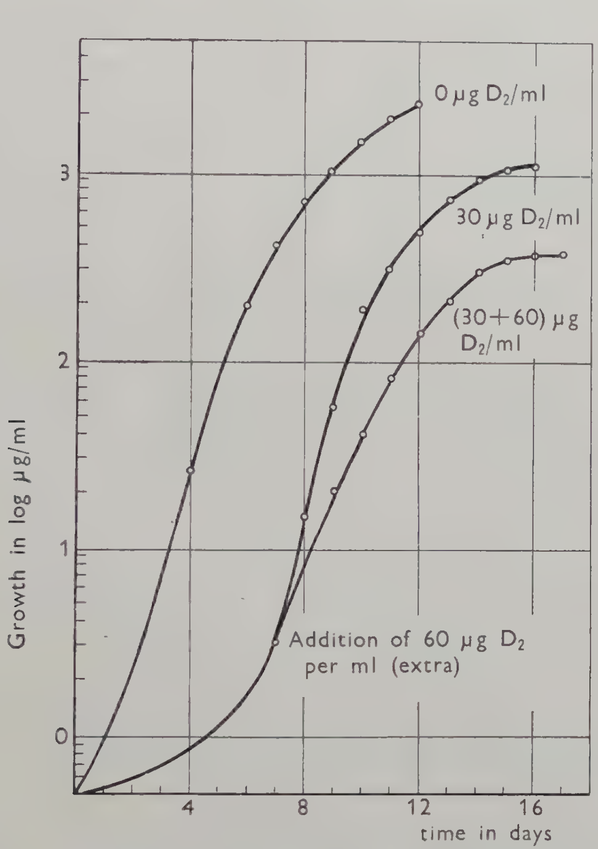


Fig. 3.
Influence of additional amounts of calciferol added after 8 days of growth of *M. tuberculosis* (strain D_{328}).

Analogous experiments with calciferol dissolved in propylene glycol and with the sodium salt of the calciferol phosphoric acid ester gave analogous results. Old (yellow) calciferol was less active. Bloodserum albumin (0.5 %) and Tween 80 (0.05 %) inhibited the action of calciferol. In their presence 175 µg/ml of the vitamin were required for an observable growth inhibition. Calciferol soon undergoes destruction in Sauton's medium. Seven days after the addition of 570 μg calciferol to 20 ml of this medium, quantitative determinations showed that only 35 μg were left [1]). The destruction

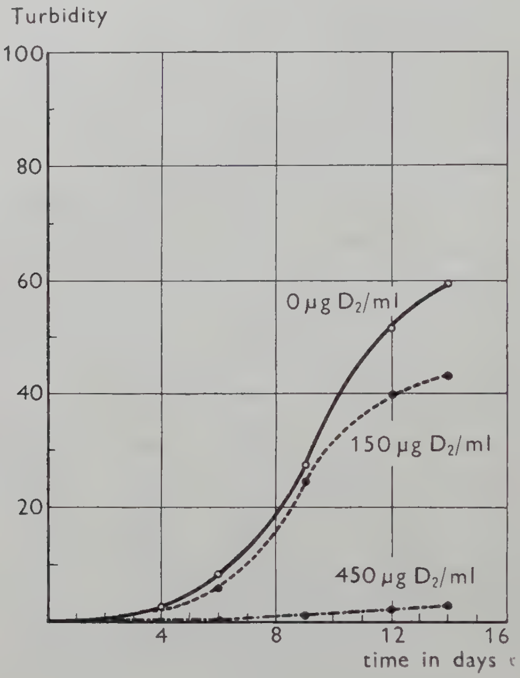


Fig. 4.
Influence of calciferol on the growth of a virulent strain (A_{30}) of *M. tuberculosis* in Dubos medium.

products are still actively inhibiting growth (fig. 2, see p. 220). This finding is of importance: if degradation products are active the low levels of vitamin D_2 in the blood of lupus patients do not necessarily exclude a direct action of calciferol (and its degradation products) on the tubercle bacteria.

Additional amounts of calciferol added after 8 days again affect the slope and the final level of the growth curve (fig. 3, see p. 221).

---

[1]) I am greatly indebted to Dr A. EMMERIE, who phosphorylated the vitamin D_2 and determined the calciferol level in the cultures.

Influence of calciferol on virulent tubercle bacteria (Strain A_{30}).

No influence was observed of concentrations up to 30 μg/ml calciferol in Dubos medium with or without serum albumin, with varying phosphate concentrations and at various temperatures (30°, 37° and 42° C.).

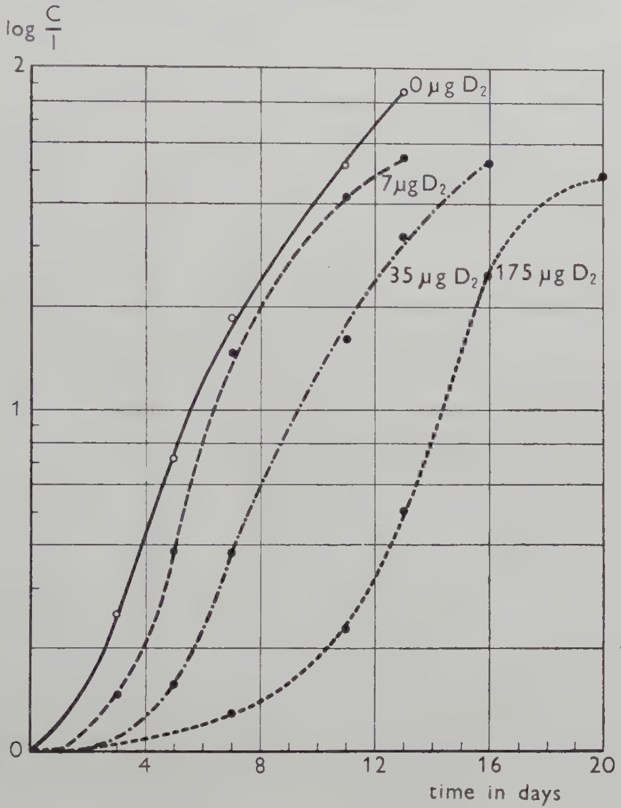


Fig. 5.
Influence of calciferol on the growth of a virulent strain (A_{30}) of *M. tuberculosis* (surface growth on the medium of Sauton).

abscis: time in days.
ordinate: logarithm of the ratio area of grown culture/area of inoculum.

The addition of calciferol dissolved in propylene glycol to the medium of Loewenstein in quantities of 5 μg/ml did not inhibit the growth of *M. tuberculosis* (A_{30}) on this medium.

Higher concentrations added to the media of Dubos and Sauton were effective however.

Fig. 4 (see p. 222) shows the inhibition of the growth of *M. tuberculosis* (strain A_{30}) in Dubos medium. A concentration of 150 μg/ml is needed. The surface growth on the medium of Sauton is inhibited by 35 μg vitamin D_2 per ml (fig. 5; see p. 223). This effect is materially reduced by the addition of 0.5% serum albumin.

Eight strains of *M. tuberculosis* isolated from lupus tissue did not appear more sensitive than other virulent strains.

The influence of other steroids on *M. tuberculosis.*

Cholesterol and ergosterol (17 μg/ml) stimulated the growth of strain D_{328} in Sauton. This is in accordance with the findings of Gernez-Rieux *et al.* (7) who reported that 250 μg/ml cholesterol promotes the growth of small inocula of virulent tubercle bacteria in the medium of Dubos.

With Vitamin D_3 equal inhibition of growth was observed to that with calciferol (strain D_{328}).

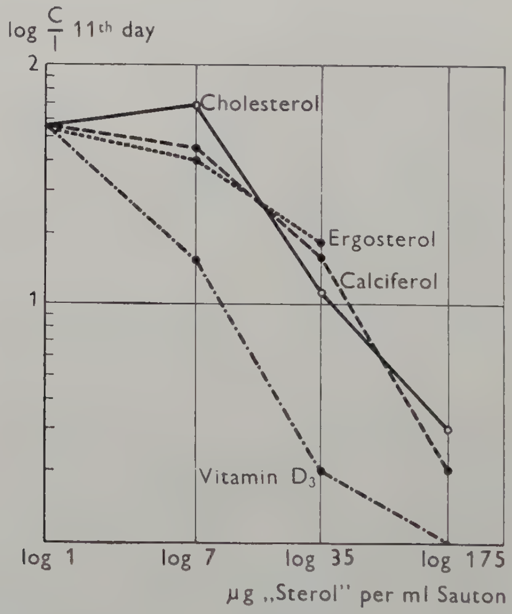


Fig. 6.

Influence of cholesterol, ergosterol, calciferol and vitamin D_3 on the growth of *M. tuberculosis* (A_{30}).

abscis: concentration sterol in μ g/ml.
ordinate: log of the ratio area of grown culture/area of inoculum as estimated after eleven days of growth.

The surface growth of a virulent strain (A_{30}) on Sauton was seriously inhibited by cholesterol, ergosterol, calciferol and vitamin D_3 (fig. 6; see p. 224). In this experiment D_3 seems more active than calciferol, this is not in accordance with other experiments and probably due to the use of an older preparation of calciferol.

The growth inhibition by vitamin D_3 is largely inhibited by the addition of 0.5% serum albumin.

Summary.

1. Calciferol and phosphorylated calciferol have a marked inhibitory action on the growth of avirulent (D_{328}) and virulent (A_{30}) tubercle bacteria in the media of Sauton and Dubos.
2. Old calciferol is less active (for clinical use freshly prepared calciferol might be of advantage).
3. Bloodserum albumin inhibits the action of calciferol.
4. Strains of *M. tuberculosis* isolated from lupus lesions did not appear more sensitive than other strains.
5. Vitamin D_3 has the same action as vitamin D_2.
6. Cholesterol and ergosterol have an inhibitory action on the growth of the virulent strain A_{30} but stimulate the diffuse growing D_{328}.

I am greatly indebted to Professor Dr K. C. Winkler for his interest, suggestions and criticism in this work.

References.

1. R. Brett and K. H. Eckes, Deutsch. med. Wsch. **74,** 859, 1949. – 2. J. Charpy, Ann. Dermat. Syph. **3,** 331, 1943; **6,** 310, 1946; Brit. J. Dermatol. **60,** 121, 1948. – 3. J. Charpy and P. Pichat, C. R. Soc. Biol. **141,** 929, 1947. – 4. I. Dainow, Dermatologica **94,** 130, 1947. – 5. L. Dickinson, Nature **159,** 581, 1947. – 6. G. B. Dowling and E. W. Prosser Thomas, Brit. J. Dermatol. **58,** 45, 1946. – 7. Ch. Gernez-Rieux, A. Sevin and Ch. Petipre-Spy, C. R. Soc. Biol. **142,** 810, 1948. – 8. M. Giroux, Ann. Rev. Biochem. **18,** 406, 1949. – 9. H. D. Groen, Thesis, Groningen 1948. – 10. A. Josserand, P. Pichat, J. Viallier and J. Kalb, C. R. Soc. Biol. **142,** 1125, 1948. – 11. W. Klip, Het vitamine D_2 en zijn werking, speciaal in verband met tuberculose. Utrecht 1948 (copies obtainable from the author); Thesis, Utrecht 1951. – 12. C. Levaditi and Li Yuan Po, C. R. Soc. Biol. **101,** 881, 1929; Presse med. **101,** 168, 1930. – 13. V. Malmström, Acta radiol. **3,** no. 19;

30–6, 1925. 14. F. Meerseman, cited by H. Gounelle, Lancet **251,** 526, 1947. – 15. P. Pichat, J. Viallier and B. Minjat, C. R. Soc. Biol. **142,** 1135, 1136, 1948. – 16. W. Raab, Science **103,** 670, 1946; Diseases of the Chest **12,** 68, 1946; **12,** 409, 1946; Science **106,** 546, 1947. – 17. Wetherly-Mein, cited by G. B. Dowling, S. Gauvain and D. E. Macrae, Brit. Med. J. **1948,** 430. – 18. Walker, cited by G. D. Powell, P. R. Pearsall and J. E. M. Wigley, Brit. Med. J. **1948,** 386.

(From the Laboratory of Hygiene and the Central Institute for Food Research, University of Utrecht, Netherlands).

THE TUBERCULOSTATIC PROPERTIES OF THE UNSAPONIFIABLE FRACTION OF COD-LIVER OIL[1]

by

W. KLIP, A. EMMERIE and CHR. ENGEL

(Received March 2, 1952).

The favourable action ascribed to the oral administration of cod-liver oil in human tuberculosis has given rise to many investigations on the influence of cod-liver oil on *Mycobacterium tuberculosis.*

Lansberg (6) found that tubercle bacteria after incubation with cod-liver oil for 45 days at 37° C. had lost their viability and showed signs of lysis. Coppens (1) has confirmed these observations.

Solomidès (7) showed that several oils lysed the tubercle bacteria. The lytic activity of cod-liver oil was weak, but a powerful bactericidal action, which increased after iodination was observed (Solomidès (8)). Oral administration to infected guinea-pigs turned the tuberculin-reaction to negative, but there was no evidence of any favourable influence on the disease. At high dosages the mean survival period of treated animals was indeed shorter than for the control group (Solomidès (9)).

We were able to show a direct bacteriostatic activity of calciferol on *M. tuberculosis* (Klip (2)). It seemed possible that cod-liver oil would contain steroids with an analogous activity to which the alleged favourable action in tuberculosis might be ascribed. To test this hypothesis the influence in vitro and in vivo of the unsaponifiable fraction of cod-liver oil on growth of tubercle bacteria has been investigated.

---

[1] This work was completed with finantial aid of the National Health Research Council (T.N.O.), the Hague, Netherlands.

Materials and methods.

Two strains of *M. tuberculosis* (type humanum) were used, an avirulent strain (D_{328}) which grew homogeneously in liquid media and a virulent strain (A_{30}) which only grew at the surface of culture media. Sauton's medium was used throughout. Growth in liquid media and surface growth was estimated as described in the previous paper.

The fractionation of the oil will be described in detail elsewhere. Briefly the procedure was the following.

Cod-liver oil was saponified and the unsaponifiable fraction extracted with ether. Sterols were removed with digitonin and the filtrate chromatographed over alumina. Elution was carried out successively with mixtures of petroleumether, benzene, benzene-ether and ether-ethanol. Only the latter fraction was growth inhibiting (fraction 5). The sterol fraction after regeneration from the digitonin precipitate had a growth-promoting activity on strain D_{328}. From 1 kg cod-liver oil about 750 mg of fraction 5 was obtained in this way. According to publications of WEIDEMANN (12), TJORBJARNARSON c.s. (11) and SWAIN (10) it is very probable that fraction 5 contains chiefly or only α-glycerylethers. Oxidation with HJO_4 and the presence of formaldehyde in the destillate supported this view.

Assuming that only substances with two OH-groups in the molecule were present, we found an average molecular weight of 356 by titration of the acid phthalic acid esters. The quantity of HJO_4 consumed by the oxidation and the amount of formaldehyde produced were in close agreement, accepting that only α-glycerylethers with an average molecular weight of 356 were present.

Results.

I. GROWTH INHIBITION OF *M. tuberculosis* BY THE UNSAPONIFIABLE FRACTION OF COD-LIVER OIL.

60 μg of fraction 5 (or of hydrogenated fraction 5) dissolved in ethanol was added to 20 ml of Sauton's medium and inoculated with 40 μg D_{328}. Control tubes obtained ethanol only. The experiment was run in triplicate. In fig. 1 growth is plotted against time.

Fraction 5 inhibits growth for a period of 8 days, afterwards growth took place as in the controls. The hydrogenated fraction is much less active. Addition of 0.05% Tween 80 or 0.5% serum-albumin to the medium largely reduced the growth inhibition (not shown).

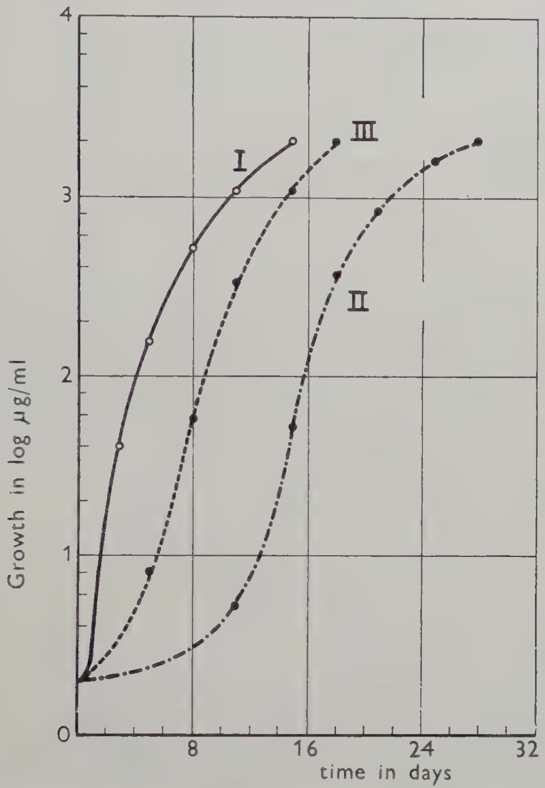


Fig. 1. Influence of fraction 5 on the growth of *M. tuberculosis* strain D_{328}.

curve I: controls.
curve II: 3 μg/ml fraction 5.
curve III: 3 μg/ml hydrogenated fraction 5.

Analogous fractions prepared by the same method from margarine, butter, yolk, lard, beef dripping and herring oil were also tested. Added in amounts of 1.5 μg/ml to Sauton's medium and inoculated with 1 μg/ml of D_{328} only the fractions of cod-liver oil. herring oil and lard delayed growth (5,4 and 1 days respectively). The other fractions were inactive.

II. MECHANISM OF THE GROWTH INHIBITION BY THE α-GLYCERYL-ETHERS OF COD-LIVER OIL.

Features of the growth curve.

Fig. 1 shows that the apparent lagtime of the growth curve is considerably increased by addition of fraction 5 to the medium, but that neither the slope of the logarithmic part of the curve nor the concentration of bacteria which were ultimately reached, were changed. After the lagtime fraction 5 seemed to have disappeared from the medium. Larger concentrations of fraction 5 gave the same type of growth curve, however, with a greater lagtime.

As incubation of the used concentration of fraction 5 in Sauton's medium (without bacteria) for ten days at 37° C. did not alter the growth inhibiting activity, these features of the curve cannot be explained by a destruction of the active substances in the medium. Of all possible explanations there were two which might come into account:

a. Neutralisation (e.g. adsorption) by the bacteria or by their metabolic products in the culture medium.

b. Destruction of the growth inhibiting substances by the organism.

The influence of dead bacteria on the growth inhibition.

To study a possible neutralisation of the active substance by bacteria or their products fraction 5 was added to two days old cultures of D_{328} (containing from 17 to 45 μg/ml of bacteria), which were then heat killed and reinoculated. Cultures without fraction 5 and ,,cultures'' with fraction 5 but without bacteria were heated and reinoculated in the same way and served as controls. No growth-inhibition could be observed in the tubes which contained 30 μg dead bacteria or more. This was taken as evidence that the active substance is neutralised by bacteria or their products.

In further experiments, varying the amounts of fraction 5 and D_{328} the neutralisation of the active substance seemed proportional to the amount of dead bacteria in the cultures. About 45 μg of bacteria seemed necessary to inactivate 1 μg of fraction 5.

In another experiment various amounts of active substance were added to cultures containing varying amounts of D_{328}. After heating the bacteria were spun down in a centrifuge and the supernate was reinoculated and incubated to test its remaining growth inhibiting activity.

Table 1.

Influence of dead bacteria on growth inhibition by fraction 5.

Number of experiment	a bacteria μg/ml	b fraction 5 μg/ml	a/b	delay of growth in days
1	210	0	~	(0)
2	260	0	~	0
3	180	3	60	0.2
4	210	4	53	0.4
5	450	10	45	0.5
6	180	4	45	0.7
7	210	5	42	0.3
8	360	10	36	1.5
9	175	5	35	0.8
10	200	6	33	0.6
11	340	11	31	1.2
12	465	15	31	2.5
13	350	13	27	1.8
14	330	15	22	3.5
15	450	25	18	8

Table 1 gives the amounts of bacteria and fraction 5 (at the moment of heating) together with the delay of growth of D_{328} in the supernate compared with control cultures. To inactivate 1 μg of fraction 5 between 30—60 μg of dead bacteria seem necessary.

The influence of metabolic products on growth inhibition.

For the case antagonists would occur in the culture medium D_{328} had to be cultured in the presence of fraction 5 and varying amounts of metabolic products. To obviate filtering or heating it was thought expedient to take cultures of D_{328} of different ages (different amounts of metabolic products), spin down the bulk of the bacteria and adding fraction 5. The bacteria remaining in the supernate served as inoculum.

A seven days old culture containing 40 μg/ml D_{328} and a twelve days old culture containing 1000 μg/ml D_{328} were used. The latter was centrifuged leaving about 80 μg/ml D_{328} in the supernate. Duplicate series of tubes involving two concentrations of fraction 5 (15 resp. 45 μg/ml) were set up with each culture. Growth was estimated at regular times. The results are plotted in fig. 2.

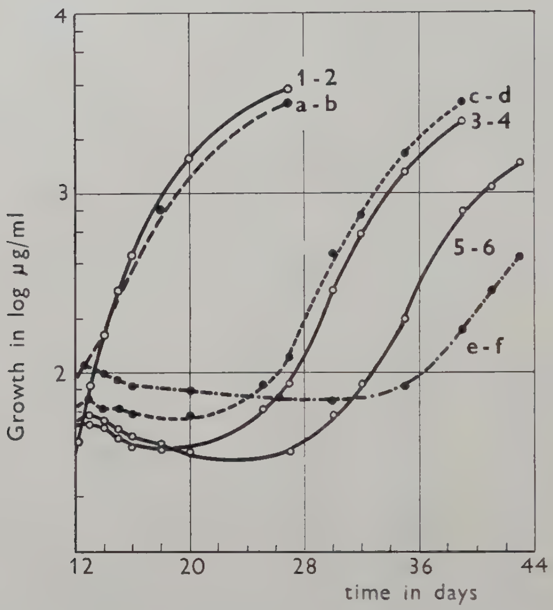


Fig. 2. Influence of metabolic products on the growth inhibition of fraction 5 on *M. tuberculosis* strain D_{328}.

1—6: cultures with small amounts of metabolic products (seven days old culture).
a—*f*: cultures with large amounts of metabolic products (twelve days old culture).
1—2, *a*—*b*: controls without fraction 5.
3—4, *c*—*d*: 15 μg fraction 5 /ml medium.
5—6, *e*—*f*: 45 μg fraction 5 /ml medium.

It is evident that the activity of fraction 5 is equal in both culture media. This is taken as evidence that no large amounts of antagonists occur in the medium of a twelve days old culture.

Fraction 5 causes a peculiar delay of growth, after which period normal growth is resumed however. This phenomenon is not due to the accumulation of antagonists in the culture medium. The phenomenon is probably due to an adsorption of fraction 5 to the bacteria. With suitable dosages the bacteria do not die but grow very slowly until the concentration of the active substance on their increasing surface no longer inhibits growth.

This supposition is in accordance with the fact that there exists a narrow relation between the number of bacteria in the inoculum and the amount of fraction 5 which inhibits growth.

The area of the bacterial surface covered by one glycerylether molecule.

The inactivation of 1 μg fraction 5 by 45 μg of dead tubercle bacteria is probably explained by an adsorption of the glycerylethers of fraction 5 to the bacterial surface. The calculation of the number of glycerylether molecules per unit of bacterial surface was thought of interest.

Length (l) and diameter (2 r) of 15 living bacteria were measured with an ocular micrometer. Taking each bacterium as a cylinder with height $h = l - 2r$ and at the end two hemispheres with radius r, the mean surface (F) and the mean volume (V) of the 15 bacteria was found to be

$$F = 9 \times 10^{-8} \text{ cm}^2 \qquad V = 2 \times 10^{-12} \text{ cm}^3$$

Total counts showed that 45μg D_{328} contained 4×10^7 bacteria with a total surface of $4 \times 10^7 \times 9 \times 10^{-8} = 3.6 \text{ cm}^2$. 1 μg of fraction 5 (MW = 356) contains 17×10^{14} molecules. Supposing — according to the experiments reported above — complete adsorption 17×10^{14} molecules were adsorbed on 3.6 cm², so one molecule took up 20 Å². The possible error of this calculation is about 60%.

The area of 20 Å² found above is interesting because KNIGHT (3) in measurements on monomolecular layers of glycerylethers in water found that one molecule of butylalcohol in the so called condensed state (15 à 16° C.) and under a surface pressure of 0 dynes covered an area of 26 Å°. In the expanded state (36° C.) an area of 60 Å² was covered under the same surface pressure. In the condensed state increase of the surface pressure compresses the area to 20 Å².

The surface covered by a glycerylether molecule is for the greater part determined by the mobility of the molecules (and consequently by the temperature) and the possibility for the molecule to take part in these movements. This mobility is smaller if the long chain of C-atoms is tied to the bacterial surface than if it moves freely among the air molecules.

Consequently the area of 20 Å² found above is of the order we would expect if the 1 μg of fraction 5 added to 45 μg D_{328} was spread out as a monomolecular layer over the bacterial surface.

This possibility seems very probable to us and we think it likely that the growth inhibiting action of fraction 5 must be due to changes in the permeability of the bacterial surface caused by the presence of this layer.

The difference in growth inhibiting activity between unsaturated and saturated glycerylethers possibly may be explained by the same reason as has been given by Kodicek and Worden (4) in the case of unsaturated and saturated fatty acids (different adsorption properties of the molecules).

III. Growth inhibition of virulent tubercle bacteria by fraction 5.

To study the question whether fraction 5 also inhibits the growth of virulent tubercle bacteria 1.5 μg and 15 μg of this fraction were added to the second and third of 3 flasks containing 25 ml of Sauton's medium each. The same was done with three flasks containing Sauton's medium with 0.5% bovine serum albumin.

The flasks were inoculated with a standard wire loop from a nine days old culture of strain A_{30} in Sauton's medium. The experiment was done in quintuple and the results are expressed in fig. 3. It appeared that without serum albumin a concentration of 1.5 μg fraction 5 per ml medium is already sufficient to cause a clear growth inhibition. However in the medium with 0.5% albumin a ten times larger concentration did not inhibit the growth.

IV. Influence of the glycerylether fraction against tuberculosis in guinea-pigs.

Forty eight guinea-pigs were infected by injecting 50 μg wet virulent tubercle bacteria (strain A_{30}, 17 days old culture on potato-medium) in the right groin. Sixteen animals received fraction 5 per os in daily doses of 25 mg enclosed in gelatine capsules. Sixteen animals were injected with a solution of 75 mg fraction 5 in arachis oil every third day. The administration of the drug began seven days before the date of infection. The remaining sixteen animals were held as controls. Fraction 5 was furthermore administrated per os to 8 healthy animals and by injection to 2 healthy animals.

The animals were weighed with regular intervals and examined for the presence of ulcera and swollen glands. Survival times were noted and the animals were autopsied as soon as possible after death. Survival periods are given in table 2.

From these data it is immediately evident that the administration of fraction 5 had a very unfavourable influence. The shortest survival period in the control group is even 50% larger than the longest period in the treated group. Results with healthy animals showed that the substance is toxic, two non infected animals died within two months after the administration with symptoms

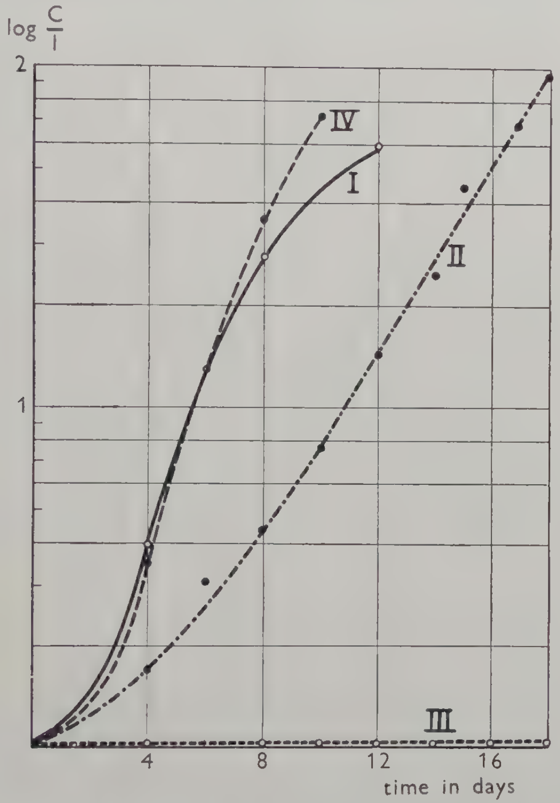


Fig. 3. Influence of fraction 5 on the surface growth of *M. tuberculosis* strain A_{30}.

abscis: time in days.
ordinate: $\log \frac{C}{I}$ (C = area of grown culture, I = area of inoculum).
I: control.
II: with 1.5 μg/ml fraction 5.
III: with 15 μg/ml fraction 5.
IV: three coinciding curves *viz.*, I, II and III with 0.5% serum albumin.

Table 2.

Number in each group	Injected with tubercle bacteria	Administration of fraction 5	Survival period in days, calculated from the date of injection		
			longest	shortest	mean
16	+	none	360	90	164
16	+	oral	59	19	34
16	+	subcutaneous	114	9	27
8	—	oral		see text	
2	—	subcutaneous	20	4	(12)

of strangulation of the intestine by connective tissue growth. The other animals in this group remained apparently healthy but showed analogous symptoms at obduction, for instance:

a. hyperaemia of intestinal mesenterium.
b. hard and swollen mesenterial lymph glands, adhering with intestine, liver and stomach by strands of connective tissue.
c. a strand of connective tissue running along the whole length of the curvatura major of the stomach and linking a packet of swollen lymphglands near the porta hepatis with the spleen.
d. intestinal adhesions.

Some workers (HIRSCH and WEINHOUSE (5)) reported the stimulation of connective tissue growth (fibroblasts accompanied by giant cells and epitheloid cells) by injection of lipids. As the glycerylethers administrated per os are probably transported in the chylolymphatic tract, the strands of connective tissue found at obduction are probably lymphvessels swollen by proliferating cells. The strands along the curvatura major of the stomach seem to support this explanation. The short survival periods of infected animals in the treated groups might be explained by the reduced resistance of the blocked and injured reticulo endothelial system.

Summary.

1. The unsaponifiable fraction of cod-liver oil contains substances which in a concentration of 2 μg per ml of Sauton's medium have a marked inhibitory action on the growth of *M. tuberculosis* D_{328} and the virulent strain A_{30}.

2. Saturated and unsaturated α-glycerylethers are very probably responsible for this activity.
3. Addition of blood serum albumin to the medium gives an important reduction of the growth inhibition by these substances.
4. With analogous fractions of margarine, butter, yolk and beef dripping growth inhibition could not be obtained.
5. The presence of dead Mycobacteria reduces the observed growth inhibition considerably. A quantitative relationship exists between the amount of dead bacteria added and the amount of inhibitor which is neutralised. It could be shown that the inhibitor is adsorbed by the added bacteria. Each bacterium binds 4×10^7 molecules.

   Supposing adsorption as a monomolecular layer, this means 1 molecule to about 20 Å^2 of the bacterial surface.
6. The glycerylether fraction of cod-liver oil (fraction 5) which inhibits in a concentration of 1 μg/ml of Sauton's medium the growth of virulent tubercle bacteria has no favourable action on tuberculosis in guinea-pigs in a daily dose of 25 mg given per os or by subcutaneous injection. Indeed the substance is toxic and oral administration to healthy guinea-pigs causes hyperaemia of the intestinal mesenterium, swollen mesenterial lymphglands and growth of connective tissue (sometimes followed by strangulation of the intestine).

The authors wish to thank Prof. Dr K. C. WINKLER and Prof. Dr H. W. JULIUS for their continuous assistance and help.

References.

1. PH. A. COPPENS, Pharm. Weekbl. **71,** 584, 1934. — 2. W. KLIP, De groeiremmende werking van vitamine D_2 en levertraan op de tuberkelbacil. Thesis, Utrecht 1951. – 3. E. KNIGHT, Biochem. J. **24,** 257, 1930. – 4. E. KODICEK and A. N. WORDEN, Biochem. J. **29,** 78, 1945. – 5. E. HIRSCH and S. WEINHOUSE, Physiol. Rev. **23,** 185, 1943. – 6. A. LANSBERG, Thesis, Leiden 1919. – 7. J. SOLOMIDÈS, C. R. Soc. Biol. **140**, 111, 1946; **140**, 838, 1946. – 8. J. SOLOMIDÈS, C. R. Soc. Biol. **143,** 751, 1949. – 9. J. SOLOMIDÈS, Ann. Inst. Pasteur **78,** 227, 1950. – 10. L. A. SWAIN, ref. Chem. Abstr. **42,** 7069, 1948. – 11. T. TJORBJARNARSON, A. SANTOZ RUIZ and J. C. DRUMMOND, Analyst **60,** 382, 1935. – 12. G. WEIDEMANN, Biochem J. **26,** 264, 1932.

(Department of Bacteriology and Experimental Pathology, Institute for Preventive Medicine, Leiden, Holland).

A MIXED EPIDEMIC OF POLIOMYELITIS AND BORNHOLM'S DISEASE (PLEURODYNIA) 1)

by

J. D. VERLINDE and **H. A. E. VAN TONGEREN**

(Received March 20, 1952).

During the first five months of 1951, a few isolated cases of poliomyelitis were recorded in the Netherlands. An increase in the incidence was noted from the month of June (23 cases), and the peak was reached in October (190 cases). At the end of May, a local outbreak of epidemic pleurodynia was reported (2), and during the next months, this disease appeared to spread over various parts of the country. Epidemic pleurodynia is not a notifiable disease, and no exact numerical data are available. While inspecting stool specimens and serum samples, which were sent to our laboratory to be examined for Coxsackie virus infection, the epidemic appeared to coincide for the greater part with the epidemic of poliomyelitis, although the peak of the former was apparently reached two months earlier (August). Similar mixed epidemics of poliomyelitis and Coxsackie virus infection (pleurodynia and "summer grippe") have been previously described (5, 4, 3).

Materials and methods

Virus isolation.

A 20 % suspension of stool specimen in saline was prepared, and centrifuged for 20 minutes at 3000 r.p.m.; the supernatant fluid was then centrifuged for a further 20 minutes at 15000 r.p.m. in a refrigerated MSE or in a Spinco centrifuge. The supernatant fluid after the last centrifuging was treated with 1000 units of peni-

---

1) Aided by a grant from the National Health Research Council T.N.O.

cillin and 2.5 mg streptomycin per ml. The material was then inoculated intracerebrally into rhesus monkeys, and either intracerebrally, intraperitoneally, or intramuscularly into suckling mice, and intracerebrally into white mice weighing 10 to 15 g. Throat-washings were centrifuged at 3000 r.p.m., then treated with penicillin and streptomycin, and inoculated into suckling mice. Blood and cerebrospinal fluid were inoculated without any treatment with antibiotics.

Poliomyelitis virus was assumed to be present when the inoculated monkeys developed flaccid paralysis, and when typical anterior horn lesions were found on histological examination of the central nervous system. Further monkey passages were made with some freshly isolated strains.

Coxsackie virus was assumed to be present when the inoculated suckling mice became paralyzed or showed convulsions, and when either muscle or fat lesions, or encephalomalacia, characteristic of Coxsackie virus, were found on histological examination, and when the agent could be serially transmitted to suckling mice, without being pathogenic for older mice following intracerebral inoculation

Typing of virus strains.

Type-specific hyperimmune sera were prepared against some of the strains isolated by us, and against the Albany types A_1, A_2, A_3, and B_1 of Coxsackie virus, which had been obtained through the courtesy of Dr G. Dalldorf (Albany, N.Y., U.S.A.). The sera were prepared in rhesus monkeys by hyperimmunization following repeated intramuscular inoculations of 5 ml of a 10 % suspension of muscle tissue from suckling mice, who showed definite signs of illness following inoculation with various types of Coxsackie virus. The type of the isolated strain was determined by neutralization tests, which were performed by subcutaneous inoculation of 10-fold dilutions of virulent muscle suspension mixed with equal parts of undiluted type-specific serum, into suckling mice. The litters used for the tests were arranged in such a way that they consisted of 8 suckling mice each. The virus-serum mixtures were inoculated into groups of 4 animals.

Attempts were also made to determine the immunological type of the isolated strains of poliomyelitis virus, the results of which will be reported later.

RESULTS

The patients examined are divided into three groups:

1. paralytic poliomyelitis,
2. aseptic meningitis,
3. pleurodynia and "summer grippe".

The majority of the patients suffering from aseptic meningitis during the poliomyelitis epidemic may perhaps be considered to be non-paralytic cases of poliomyelitis, and many of those suffering from summer grippe may perhaps be considered as cases of abortive or extraneural poliomyelitis. Clinically, the differential diagnosis between pleurodynia and minor illness characterized by gastro-intestinal disturbances, sore throat, pain in muscles and joints (summer grippe) may be difficult or impossible. Both these syndromes are, perhaps, different grades of the same disease, and may be due to infection either with poliomyelitis virus, or with Coxsackie virus, or with both of them, as has been shown by SABIN and STEIGMAN (5), and MELNICK and his associates (3).

All patients were examined for the presence of Coxsackie virus, usually in stools, and occasionally in blood and cerebrospinal fluid. Seventeen strains of Coxsackie virus were recovered from stools, and one from the blood. No evidence of the presence of Coxsackie virus in muscle biopsy material from a poliomyelitis patient was obtained, although both poliomyelitis and Coxsackie virus were isolated from the stools. Neither herpes virus, nor choriomeningitis virus was isolated from the blood or the cerebrospinal fluid of patients suffering from aseptic meningitis.

Twenty patients suffering from paralytic poliomyelitis were examined for poliomyelitis virus. In 6 cases (30 %) poliomyelitis virus only, in 1 case (5 %) Coxsackie virus only, and in 2 cases (10 %) both poliomyelitis and Coxsackie virus were isolated from the stools.

Table 1 shows that 5 types of Coxsackie virus, in which the Albany A_2 type dominates, have been isolated from approximately the same percentage of patients in the three different groups. The types V and W were unrelated both to the Albany types used in this investigation, and to one another. Of two additional strains, isolated by Dr F. DEKKING (Amsterdam), one appeared to be related to the Albany A_2 type, and one to the Albany B_1 type, so that at least 6 types of Coxsackie virus were isolated in the Netherlands during the mixed epidemic of 1951.

Table 1.

Percentage and type of Coxsackie virus isolations.

Group of patients	percentage		type					
	year 1951	July to Oct.	A_1	A_2	A_3	B_1	V	W
paralytic poliomyelitis	11 %	25 %	1	4				1
aseptic meningitis	14 %	20 %	1	2	1			
pleurodynia and summer grippe	11 %	16 %		7			1	

In 7 patients, 2 of whom were suffering from paralytic poliomyelitis, 2 from aseptic meningitis, and 3 from pleurodynia, the development of neutralizing antibodies against the type of virus that had been recovered from their stools, could be demonstrated. In one patient suffering from paralytic poliomyelitis, and from whose stools both poliomyelitis and Coxsackie virus were isolated, no development of Coxsackie-antibodies could be shown.

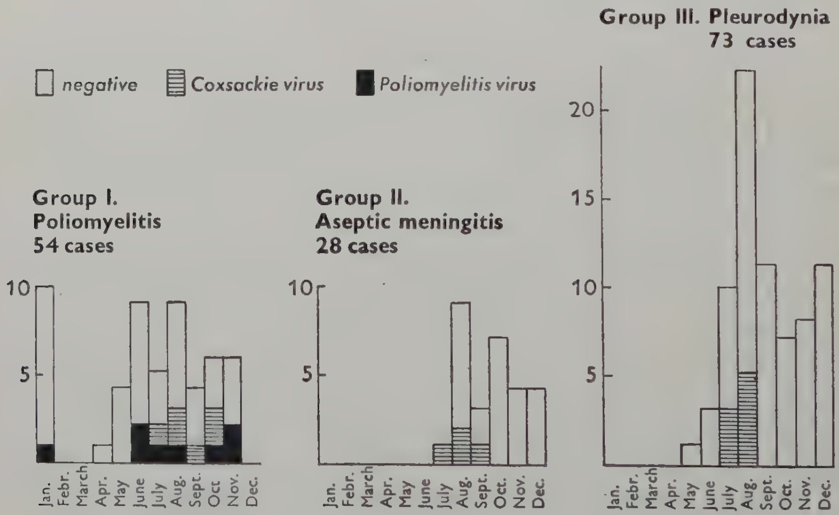


Fig. 1. Distribution of the cases examined.

Figure 1 shows the distribution of the cases examined. It should be noted that their number is not in accordance with the number of cases that actually have occurred, or, as far as poliomyelitis is concerned, have been recorded. As a matter of fact, the peak of the poliomyelitis epidemic was reached in October, which cannot be read from this figure.

Isolation of Coxsackie virus from a monkey.

Coxsackie virus of the Albany A_2 type was isolated from the stools of a patient suffering from paralytic poliomyelitis, and a rhesus monkey developed paralysis following intracerebral inoculation of the same stool specimen. Typical anterior horn lesions were found, and in the spinal cord a poliomyelitis virus was present, which could readily be transmitted from monkey to monkey in three subsequent passages. From the stools of the monkey of the first passage Coxsackie virus of the same type as that in the patient's stool specimen was isolated, which seems to indicate that Coxsackie virus inoculated intracerebrally into the rhesus monkey may be excreted through the faeces.

A newborn rhesus monkey was inoculated intramuscularly with this strain (suckling mouse muscle), but no symptoms developed, and no lesions could be shown in muscle biopsy material.

Discussion.

Coxsackie virus is the only pathogenic agent found so far in pleurodynia, and the development of antibodies to it would indicate that it may be considered to be the causative agent. The same virus, however, has also been frequently isolated in cases of poliomyelitis and the development of antibodies has been shown, so that a mixed infection with both poliomyelitis and Coxsackie virus does not seem to be an uncommon occurrence. It has been considered that both Coxsackie and poliomyelitis virus, when infecting man simultaneously, might influence one another, either antagonistically, or in promoting paralysis. DALLDORF's (1) experiments in which a sparing effect of Coxsackie virus on the development of paralysis, and the survival of mice following inoculation of the Lansing strain of poliomyelitis virus, were observed, might point to some antagonism or interference, whereas a recent paper of MELNICK and his associates (3) might be suggestive of a promoting effect of Coxsackie virus on the development of paralysis. In the mixed epidemic described in the present paper, no convincing evidence has been obtained of either a sparing or an enhancing effect on the development of paralysis by Coxsackie virus infection.

As a matter of fact, the percentage of Coxsackie virus isolations in paralytic poliomyelitis was higher than in non-paralytic poliomyelitis and in pleurodynia during the epidemic months from July to October; but the fact that stools from patients suffering from pleurodynia have frequently been sent for virological examination at the end of the disease, whereas stools from poliomyelitis patients usually have been examined in the first few days of the paralytic stage, should be taken into consideration.

The pathology of Coxsackie virus infection in man is unknown. If the virus, however, produces muscle lesions, which in man are presumably much less severe than in suckling mice, it is not unlikely that this may enhance the development of paralysis in a combined infection with poliomyelitis virus. It seems generally accepted that a lowering of the resistance of skeletal muscles, e.g. by fatigue, intramuscular inoculations (pertussis vaccine and diphtheria toxoid A.P.T.) may provoke the occurrence of paralytic poliomyelitis in carriers of the poliomyelitis virus. Coxsackie virus, certain strains of which are presumably also myotropic in man, may exert a similar resistance-lowering effect, the mechanism of which still calls for an explanation.

Summary.

During a mixed epidemic of poliomyelitis and Bornholm's disease in the summer of 1951, evidence was obtained of the involvement of at least 6 different immunological types of Coxsackie virus, among which the Albany A_2 type dominated. Poliomyelitis virus was isolated from the stools of 6 out of 20 patients suffering from paralytic poliomyelitis; Coxsackie virus from 1, and both poliomyelitis and Coxsackie virus from 2 out of these 20 patients. During the whole year, Coxsackie virus was recovered from the stools of patients suffering from paralytic poliomyelitis, aseptic meningitis, pleurodynia and summer grippe in approximately equal percentages (11 to 14 %), but during the epidemic months from July to October, 25 % of the patients with poliomyelitis, and 16 % of the patients with pleurodynia gave positive results for Coxsackie virus. The sparing or the enhancing effect of Coxsackie virus infection on the development of paralysis in patients with dual infections is discussed.

We are indebted to Miss O. J. A. CALMEYER and Miss N. FILIPPO for their technical assistance.

References.

1. G. DALLDORF, Journ. exp. Med. **94**, 65, 1951. – 2. L. FAÇEE SCHAEFFER, Ned. Tijdschr. v. Geneesk. **95**, 2938, 1951. – 3. J. L. MELNICK, A. S. KAPLAN, E. ZABIN, G. CONTRERAS and N. W. LARKUM, Journ. exp. Med. **94**, 471, 1951. – 4. J. L. MELNICK, N. LEDINKO, A. S. KAPLAN and L. M. KRAFT, Journ. exp. Med. **91**, 185, 1950. – 5. A. B. SABIN and A. J. STEIGMAN, Amer. Journ. Hyg. **49**, 176, 1949.

(From the Laboratory for Hygiene of the University of Amsterdam).

THE SUSCEPTIBILITY OF *SPIRILLUM MINUS* AND *BORRELIA DUTTONI* TO STREPTOMYCIN AND PENICILLIN

by

J. BIJLMER

(Received January 23, 1952).

After reading the article by JELLISON and co-workers in the Public Health Reports of 1949 (1), which described the successful treatment with streptomycin of a girl suffering from ratbite fever, we made several tests on mice and guinea pigs. Meanwhile, a few observations of similar purport have been published (2).

Altogether 398 mice were treated by us with streptomycin or penicillin after being infected with *Spirillum minus*. The lowest dosage was a single injection of 0.025 mg streptomycin, given to 2 mice. The peritoneal liquid of both mice remained free from Spirilla for 4 days (at least according to our examination with dark-field illumination). On the 5th day the result proved to be positive again.

Higher but single dosages did not cure the mice from their infections either. The lowest dosage with which mice were cured was 0.1 mg streptomycin daily for 4 days. This treatment proved to be successful in 3 out of 6 mice. Not one of the 57 controls, which did not get any treatment, became negative. The mice were checked for at least 6 weeks after treatment. Those which became positive again after being treated usually did so after about 3 weeks; all the relapses occurred within 30 days after the last day of treatment, with one exception, which took 35 days to become positive again. The mice which according to our dark-field examinations were definitely cured, were at first checked once or twice by subinoculation tests. They proved that our dark-field examination was fully reliable.

The results obtained with higher dosages and prolonged treatments were better, but did not nearly come up to expectation, the percentage of cures never exceeding 85. A curious fact was that above a certain dosage the results became poorer rather than better.

Of 46 mice treated daily with 0.2 mg streptomycin for 6 days 11 (about 24%) kept their infection, while 35 recovered. For 105 mice which received 0.3 mg daily for 6 days, these figures were 26 (about 25%) and 79, respectively, thus practically identical.

These figures were rather less favourable (36 or about 32% failures and 77 recoveries) for 113 mice which were given 0.4 mg daily for 6 days. A daily dosage of 0.5 mg during 6 days resulted in 3 failures with 11 mice; 0.6 mg per day for 6 days gave 6 failures in 13 mice.

When streptomycin was administered for less than 6 days at a stretch, the results dropped, but when the period of treatment was not shorter than 4 days and the dosage not less than 0.1 mg per day, the percentage of recoveries was always 50 or more (110 mice were treated with dosages varying between 0.1 mg per day during 4 days and 0.4 mg per day during 5 days). A more prolonged therapy yielded somewhat better results; yet, as already stated, they did not come up to our original expectations. A therapy of 0.2 mg of streptomycin daily during 2 weeks (the Sunday excepted) proved successful in 12 out of 14 mice; 0.3 mg daily in 7 out of 9 mice. These figures, when added up because of the small numbers involved, result in 4 failures out of 23 cases (hence no less than over 17% failures).

As a patient treated with streptomycin for bacterial infections can develop streptomycin resistance within a notoriously short time, a few tests in this direction were carried out. 16 Mice which had relapsed after a week's treatment with 0.3 or 0.4 mg of streptomycin per day were treated a second time for one week with dosages varying between 0.4 and 0.6 mg per day. Not one of them was freed from its *Spirillum* infection. Indeed, after the second treatment the Spirilla were detected sooner in the peritoneal liquid than after the first: after the first cure the period between the last day of treatment and the first day of Spirilla detection in the dark-field examination was seldom shorter than 2 weeks; after the second treatment it was usually shorter than 2 weeks.

Penicillin tests, on the other hand, yielded satisfactory results. Although at first penicillin seemed to be less active — Spirilla being detected in the peritoneal liquid, for instance already one day after a dose of 100 units — further experiments showed that high dosages of penicillin were absolutely effective.

Of 28 mice which received 500 units daily for 6 days, 26 became permanently negative. All the 53 mice which were given 1000 units daily were freed from their infection. With the penicillin therapy it did not matter at all whether or not the mice had already been treated with streptomycin. Thus, one of the 2 failures after 6 × 500 units of penicillin recovered quickly after a second cure of 6 × 1000 units. This one case does not, of course, allow of any conclusions being drawn as regards the development of penicillin resistance.

Guinea pigs proved to constitute an entirely different case. The infection was much more virulent. The males developed orchitis and ran a fever, to which they ultimately succumbed. However, streptomycin therapy offered a remedy here, although as compared with the mice low dosages were administered for a short period [1]).

Five guinea pigs were given 2 mg daily for 4 days; one received 3 mg for 3 days; one 4 mg daily for 3 days; one 8 mg daily for 3 days; three only got 5 mg daily during 2 days.

All the 11 guinea pigs recovered, the orchitis disappeared, the temperature dropped and no Spirilla could be found any more. A really outstanding success was achieved with 3 of these guinea pigs, which were so ill that any effort to cure them seemed quite useless. All the 7 untreated guinea pigs died.

The two hamsters used as experimental animals came closer to the mice in this respect than to the guinea pigs. One was daily given 2 mg streptomycin for 4 days, the other for 3 days (weight 100 and 90 grams, respectively). Both became positive again and one died comparitively shortly after. The other one was definitely freed from Spirilla by 2000 units of penicillin daily during 3 days.

We conclude that with some species of animals streptomycin can be used to treat *Sp. minus* infections, with others it cannot. Penicillin is perhaps a good remedy with all kinds of animals, but the tests described are still too incomplete for a definite opinion to be given.

Both penicillin and streptomycin gave absolutely unsatisfactory results against *Borrelia duttoni* infections in mice. With streptomycin dosages of 0.2 to 0.4 mg per day during 6 days a successful result was obtained only in 7 out of 55 mice; with 1000 units of penicillin per day 6 out of 10 mice retained their infection.

---

[1]) The mice weighed about 20 g, the guinea pigs between 300 and 400 g.

As has been mentioned above, a few cases have already been described in the literature in which patients with ratbite fever recovered quickly with streptomycin in a dosage of 1 g per day during 4 to 10 days. As regards the susceptibility of *Spirillum* infection to streptomycin fortunately, human beings are more closely related to the guinea pig than to the mouse; at least, the few observations hitherto made point in this direction. It may be interesting to note in this connection that *Sp. minus* infections cause a serious illness in human beings as well as in guinea pigs, which is not the case with similar infections in mice and hamsters.

Published results of experiments on this subject are scarce. In 1943, LOURIE and COLLIER (3) made penicillin tests on mice with *Sp. minus* infections. They obtained much better results than we did! They were successful with 10 × 10 units distributed over 2 days or with the administration of 1000 units at once; an achievement we have never been able to attain.

Much more attention has been paid to *Borrelia* infections, which indeed are of more practical importance. Prophylactically, both penicillin and streptomycin were useful with mice, but the therapeutic results were much more unsatisfactory, unless the antibiotic was applied intracerebrally (4, 5).

The statements about human infections vary greatly, but most investigators come to the conclusion that the antibiotics mentioned are of no avail, at least against *B. duttoni* infections (6).

According to the most recent data, aureomycin is suitable as well as active — also in the case of Sodoku — but we have not yet experimented with this antibiotic.

Summary.

An investigation into the effect of streptomycin and penicillin on *Spirillum minus* infections in mice, guinea pigs and hamsters, showed that streptomycin effectively controlled *Spirillum* infections in guinea pigs, but that this antibiotic was not always efficacious in mice and hamsters. Penicillin, when given in rather high dosages, seemed to be more reliable.

Both antibiotics were absolutely inadequate for the control of *Borrelia duttoni* infections in mice.

References.

1. W. L. Jellison, P. L. Eneboe and R. R. Parker, Publ. Hlth Rep. **64,** 1661, 1949. –2. H. Floch, Y. Constant and P. Destombes, Bull. Soc. Path. Exot. **43,** 406, 1950. – 3. E. M. Lourie and H. O. J. Collier, Ann. Trop. Med. Paras. **37,** 200, 1943. – 4. E. S. Anderson, Tr. R. Soc. Trop. Med. a. Hyg. **40,** 93, 1946. – 5. C. Levaditi and A. Vaisman, C. R. Soc. Biol. **140,** 29, 1946; C. R. Acad. Sciences **225,** 769, 1947. – 6. R. Néel, M. Payet and C. Gonnet, Bull. Soc. Path. Exot. **42,** 384, 1949.

(Department of Bacteriology and Experimental Pathology of the Institute for Preventive Medicine, Leiden Holland).

HAEMATOGENOUS SPREAD OF POLIOMYELITIS VIRUS[1]

by

J. D. VERLINDE and **B. BEEM**

(Received April 22, 1952).

It is almost generally accepted that, when poliomyelitis virus spreads through a population, it causes subclinical and clinically unrecognizable abortive infections in the majority of the cases. Only a relatively small proportion of the infected individuals contract paralytic disease. The abortive cases which may show various signs of minor illness, i.e. sore throat, gastrointestinal disturbances, muscle pain ("summer grippe": SABIN and STEIGMAN (9)), and the non-paralytic meningitic forms, are essentially extraneural forms of poliomyelitis because the virus has been recovered from them.

At the Third European Poliomyelitis Conference, held in 1950 at Amsterdam and Leiden, MOLLARET (8) insisted on the myotropic properties of poliomyelitis virus. In his view, many patients treated in the artificial respirator do not die from respiratory paralysis, but from myocarditis. As a matter of fact, myocardial lesions have not infrequently been found in poliomyelitis patients at autopsy. The occurrence of severe pain in certain muscle groups, e.g. those of the neck and the back, might also point to myositis (LARUELLE (7)).

Since JUNGEBLUT and coworkers (5, 6) succeeded in isolating poliomyelitis virus from the skeletal as well as from the heart muscle of fatal cases, and JUNGEBLUT (3) and VERLINDE and his associates (10, 12, 13) produced myocardial and skeletal muscle lesions in monkeys with members of the Columbia SK group of viruses as well as with classical poliomyelitis virus, a muscular

---

[1] Aided by a grant from the Dr Simon Baruch Foundation and from the National Health Research Council T.N.O.

involvement in poliomyelitis seems to be in the realms of possibility.

The extraneural distribution of poliomyelitis virus which occurs essentially during the initial phase of poliomyelitis infection, and which may result in minor illness, myositis, myocarditis, and meningitis, is difficult to explain by exclusive neural spread of the virus. One of the possible routes of non-nervous spread would be via circulation. If viraemia occurs, one would expect it to occur long before the paralytic stage, presumably in the initial phase, or, even during the early incubation period. Although viraemia during the first four days of the incubation period has been demonstrated in monkeys inoculated intramuscularly with members of the Columbia SK group of viruses (10, 13), we could not show a haematogenous spread in a small-scale experiment with the A.K. strain of poliomyelitis virus. The reason for this failure was perhaps the relatively low virulence of the strain, which might be diluted beyond the minimal PD_{50} dose in the blood stream. Therefore, we have made similar attempts with the Leon strain of poliomyelitis virus [1]), which brings down monkeys regularly upon intramuscular inoculation. Practically 100 % of monkeys inoculated by this route with 1 ml of a dilution of 10^{-1} to 10^{-2} of monkey cord come down with paralysis, showing typical anterior horn lesions.

EXPERIMENTAL.

A group of 8 rhesus monkeys was inoculated into the thigh or calf muscles with 1 ml of a 10 % suspension of monkey cord having a PD_{50} titre between 10^{-6} and 10^{-7}. All of these animals were bled from the heart at daily intervals, and 6 animals were killed at different days following inoculation. The blood was collected in sodium citrate, centrifuged for 30 minutes at 3000 r.p.m., and the plasma was inoculated both intracerebrally (1 ml) and intramuscularly (10 to 40 ml) into rhesus monkeys. The sedimented blood cells were haemolyzed by adding sterile distilled water, and then centrifuged for 1½ hour at 40.000 r.p.m. in an ultracentrifuge (Spinco). The supernatant fluid was carefully removed, leaving 1 ml at the bottom of the tube, which was then inoculated intracerebrally into rhesus monkeys. Moreover, 10 % suspensions, in distilled water, were prepared from the inoculated muscle, the corresponding sciatic nerve, and the inguinal lymphnodes. The latter

---

[1]) The Leon strain was kindly supplied by DR H. A. HOWE, Baltimore Md. U.S.A.

Table 1.

Inoculation of monkeys.

Monkeys inoculated i.m. with Leon virus		bled or killed following inoculation	transfer of blood or tissues									
			blood plasma		blood cells		muscle		sciatic nerve		inguinal lymphnode	
No.	onset of paralysis		monkey	onset of paralysis	monkey	onset of paralysis	monkey	onset of paralysis	monkey	onset of paralysis	monkey	onset of paralysis
A 455	killed 1st day	1 day			B 404	—	B 405	8th day	B 406	—		
A 397	killed 2nd day	2 days			B 408	18th day	B 409	8th day	B 410	—		
A 398	killed 3rd day	3 days			A 463	17th day	A 464	—	B 407	13th day		
A 402	killed 5th day	5 days			B 411	17th day	A 465	10th day	A 466	—		
A 403	killed 7th day	7 days			A 476	—	A 468	8th day	A 469	—	A 470	17th day
A 371	paralysis 8th day	1 day	A 395	—	B 396	17th day						
		3 days	B 402	—	B 403	—						
		5 days	A 406	—	A 407	—						
		7 days	A 410	—	A 411	—						
A 372	paralysis 9th day	2 days	B 397	—	B 398	—						
		4 days	A 404	—	A 405	—						
		6 days	A 408	—	A 409	—						
A 454	paralysis 8th day	9 days					A 471	—	A 472	—	A 473	13th day

were chosen because of the positive results obtained by Wenner and Rabe (14) with the inoculation of lymphnode material from human poliomyelitis cases. The results of the experiments are presented in table 1.

It appears, that blood plasma failed to produce paralysis, which indicates that the virus is not present in this fraction, or may have been contained in too low a concentration to produce paralysis. Five of 6 monkeys, however, which had been inoculated with the sedimented remnants of haemolyzed blood cells taken on the first, second, third, and fifth day following inoculation, developed typical paralysis. In all of these monkeys typical anterior horn lesions were found, which corresponded with unilateral paralytic involvement. The incubation period of 17 and 18 days is unusually long for Leon virus, which may indicate, that the virus concentration was relatively low. These results indicate that the virus was actually associated with the cellular material of the blood.

The presence of large amounts of virus in the inoculated muscles during the first 7 days following inoculation was a surprising observation. The monkeys inoculated with muscle suspension developed paralysis and typical anterior horn lesions after an incubation period of 8 to 10 days. It clearly indicates that the virus not only survives in the muscle, but multiplies in this tissue. This is in harmony with the results of virus growth in tissue cultures by Enders and his associates (2). It also agrees with the clear-cut muscle lesions found in the intramuscularly inoculated monkeys in this experiment and in earlier work. Of further interest is the fact that most of the inoculated monkeys showed definite myocardial involvement.

Although the presence of virus in the sciatic nerve could be demonstrated in only one of the 6 intramuscularly inoculated animals, there is sufficient evidence that also in the Leon virus infection axonal spread occurs. The presence of virus in the inguinal lymphnodes before and after onset of paralysis agrees with the observations of Wenner and Rabe (14).

COMMENT.

The experiments described leave little doubt that classical poliomyelitis virus, at least the Leon strain, may be transported through the body by the blood stream during the incubation period. In this respect, there is no longer an essential difference between the pathogenesis of this virus infection and that of infection with viruses of the Columbia SK group. The haematogenous distribution of the virus explains many symptoms and lesions outside the central nervous system during the initial stage of poliomyelitis, and during forms of extraneural poliomyelitis, which are never followed by paralysis. One of the most far-reaching and important conclusions, which may be drawn from these results, provided they can be confirmed with other types of poliomyelitis virus, deals with the possibility of active immunization. If peripheral infection precedes invasion of the central nervous system, it is conceivable that with the production of circulating antibodies the haematogenous spread of the virus may be checked. Therefore it seems unnecessary to insist on the production of a solid immunity of the central nervous system, which is extremely difficult to obtain, at least when using inactivated virus. Moreover, passive immunization by immune serum or by gamma globulin, as reported by BODIAN (1), might be of some value in preventing paralysis when applied early in the incubation period, i.e. at a time when virus is still in the blood stream.

In accordance with views expressed by JUNGEBLUT (4), presumably the following suggestions as to the concept of poliomyelitis could be made. The virus, when infecting man naturally by oral route, may multiply in the gastrointestinal tract and produce minor gastrointestinal disturbances. From here, a haematogenous spread seems to be possible, resulting in muscular (skeletal muscles and myocardium), and perhaps meningeal involvement. A final neural spread, from whatever tissue occurring, may eventually produce paralytic disease by involvement of the motor nerve cells of the central nervous system. The vast majority of the infected individuals show only signs of extraneural poliomyelitis.

This concept might also furnish an explanation for the occurrence of poliomyelitis following prophylactic inoculations, e.g. with pertussis vaccine and diphtheria toxoid. If virus has been transported by the blood stream to muscle or other tissues, a local damage as induced by intramuscular inoculation might conceivably facilitate its neural spread and the subsequent development of paralysis (VERLINDE (11)).

Summary.

In rhesus monkeys inoculated intramuscularly with the Leon

strain of poliomyelitis virus, the agent could be recovered from the blood during the first five days of the incubation period. It was apparently associated with the blood cells. It was also demonstrable in the inoculated muscle during the first 7 days. Moreover, virus was present in the sciatic nerves and in the regional lymphnodes. The significance of these findings in relation to the mechanism of infection, and in relation to the possibility of active or passive immunization is discussed.

References.

1. D. Bodian, Amer. Journ. Hyg. **54**, 132, 1951. – 2. J. F. Enders, Paper presented before the Second International Poliomyelitis Conference, Copenhagen, 1951. – 3. C. W. Jungeblut, Bull. N.Y. Acad. Med. **26**, 571, 1950. – 4. C. W. Jungeblut, Journ. Pediatr. **37**, 109, 1950. – 5. C. W. Jungeblut and J. E. Edwards, Amer. Journ. Clin. Path. **21**, July, 1951. – 6. C. W. Jungeblut and M. A. Stevens, Amer. Journ. Clin. Path. **20**, 701, 1950. – 7. L. Laruelle, personal communication 1952. – 8. P. Mollaret, Le démembrement de la poliomyélite. Masson, Paris, 1950. – 9. A. B. Sabin and A. J. Steigman, Amer. Journ. Hyg. **49**, 176, 1949. – 10. J. D. Verlinde, Arch. Virusforsch. in print. Presented in the discussions before the Second International Poliomyelitis Conference, Copenhagen, 1951. – 11. J. D. Verlinde, Arch. Virusforsch. in print. – 12. J. D. Verlinde, P. de Baan and J. A. Vercruysse, Antonie van Leeuwenhoek **16**, 9, 1950. – 13. J. D. Verlinde and B. Hofman, Arch. Virusforsch. in print. – 14. H. A. Wenner and E. F. Rabe, Amer. Journ. Med. Sciences **222**, 292, 1951.

(From the Rijks Instituut voor de Volksgezondheid, Utrecht, Holland).

THE FORMATION OF BACTERIOPHAGES IN MIXED CULTURES BY RECOMBINATION OF GENETIC ELEMENTS; CHARACTERISATION OF PHAGE TYPES OF *SALMONELLA PARATYPHI* B BY PHAGE REACTIONS AND LYSOGENIC PROPERTIES

by

R. TH. SCHOLTENS

(Received March 31, 1952).

The discovery of the Vi antigen by Felix and Pitt (8) has given an impulse to the development of knowledge in another field, the field of bacteriophage. Craigie and Brandon (2), Sertic and Boulgakov (19) and Scholtens (17) demonstrated that specific phages exist attacking only those strains of *S. typhi* that contain the Vi antigen.

A special property of a serological subgroup of these phages allowed Craigie and Yen (5) to develop the method of phage typing. When propagated upon a given strain of *S. typhi* these Vi bacteriophages develop a specific affinity for this very strain. Higher dilutions of the preparations obtained attack this strain, and strains from the same outbreak, and other strains considered therefore to belong to the same type. These higher dilutions are utilised so as to recognise the strains of the same type. The specifity of the preparations is such, that type after type, and preparation after preparation could be added to the system, without affecting the classification of the strains belonging to types already recognised. Up till now 29 types have been found.

It appears that the factors underlying the difference between the types rule also the affinity of the other (Vi) bacteriophages for (typhoid) bacteria. These bacteriophages each attack a different combination of types. Inversely, each bacterial type is attacked by a different combination of phages out of a given set and can be recognised by these reactions.

It is clear that by the addition of a new phage to such a system not only bacterial strains, which did not react with any of the phages already utilised, can be recognised, but also a further subdivision of types already identified may be found.

Systems consisting of a set of unadaptable phages have been utilised successfully for typing purposes with various bacterial species e.g. *Staphylococcus aureus* [Fisk (10), Fisk and Mordvin (11); Wilson and Atkinson (21)], *S. typhi murium* [Lilleengen (12)].

In most countries for typing *S. paratyphi* B the system of Felix and Callow (7, 9) is used. This system consists partly of preparations obtained by adaptation of a given bacteriophage, and partly of other phages attacking several types. The preparations are distributed by Dr Felix from the Central Reference Laboratory to laboratories in different countries. In this way, in all countries typing is performed with the same dilutions of exactly the same preparations. There is no need to explain here the immense value of this procedure.

A second group of phenomena depending on the mentioned principle underlying sensitivity to bacteriophage is spontaneous lysogenicity. Otto and Munter (14) isolated phages from pure bacterial cultures. Lisbonne and Carrère (13) incubated mixed cultures of two different strains and demonstrated the appearance of bacteriophages. A relation appeared to exist between the phages produced by spontaneous lysogenicity and bacterial types found by phage reactions. Craigie (3) showed strains of type D_1 of *S. typhi* to be lysogenic. Boyd (1) demonstrated that strains of *S. typhi murium* can be subdivided in types characterised by the phages produced spontaneously. For *S. paratyphi* B it was found that a relation existed between "phagetypes" as recognised by phage reactions, and the characteristics of the phage produced by lysogenicity [Scholtens (18)]. This was also shown by Felix and Callow (9) and Nicolle and d'Yves Hamon (15).

Moreover, it could be demonstrated that types of *S. paratyphi* B can be characterised by lysogenicity and phage reactions [Scholtens (18)]. One of these types was a type "Midwoud" (the same strains have been typed "Beccles" by Felix).

In filtrates of type "Midwoud" only a single bacteriophage

phage I (Scholtens) (phage 3b, Felix) can be demonstrated. If, however, these bacteriophages are incubated in mixed culture with strains of other types, phages which are not found in the strains when grown separately, are seen to appear after a five days' incubation. Here a relation between the characters of the bacteriophages found and the respective types of the strains grown in mixed culture will be demonstrated. It will be shown that these phages are formed by a recombination of (phage) elements present in the respective strains.

TECHNIQUE.

1. Nutrient media.

In all experiments a medium prepared with fresh meat described by Scholtens (17) was utilised. This medium contains bactopepton and meat in the same proportions as the standard medium recommended by Craigie and Felix (4).

2. Titration of phage preparations.

The technique of Craigie, Felix and co-workers (4, 5) was followed throughout.

3. Isolation of bacteriophages from mixed cultures.

After a week's incubation at 37° C. small quantities (2 or 3 ml) of the mixed cultures were heated in a water bath at 56° C. during 30 minutes. A drop of each of a series of dilutions was brought on a nutrient agar medium inoculated with a broth culture of the sensitive strains. After 24 hours incubation at 37° C. a single plaque was touched with a platinum needle and the inoculum was brought on the surface of a nutrient agar medium inoculated with a broth culture of the sensitive strains. Now the greater part of the surface is flooded again with the infected condens water. After 24 hours' incubation a part of the medium covered with bacteria and numerous plaques was brought into broth incubated for 18 hours. This preparation, or preparations obtained after successive subcultures from separate plaques were heated at 56° C. during one hour, titred and examined in the critical test concentration for its host range and serological properties.

4. Host range.

The critical test concentration is examined on a set of strains on the agar medium described.

5. Serological investigations.

0.3 ml of each of a series of twofold dilutions is added to an equal quantity of the phage preparation to be examined. The dilution of the phage preparation was such as to produce semiconfluent lysis (or in the case of very minute plaques, a great number, 100 till 500, of plaques in the experimental conditions).

After staying an hour in the water bath and 18 hours at room temperature, a drop of each dilution is brought on the surface of an agar plate inoculated with a sensitive strain. Readings are made after 8 or 24 hours. A positive effect is noted when plaques do not appear at all, or in greatly diminished numbers in comparison with a control.

DESCRIPTION OF BACTERIOPHAGES.

The bacteriophages demonstrated in the different types by Scholtens (17, 18) were indicated by Roman numerals.

Phage I. This phage corresponds with the bacteriophage 3b of Felix and Callow (9). It possesses characteristic plaques of $^1/_3$ to $^1/_2$ mm diameter, with an irregular edge. The secondary growth is abundant, also with an irregular edge, and a smooth surface. When concentrated preparations are being examined not only the plaques but also the secondary growth becomes confluent and lysis can be seen only as an irregular ring. Halo is generally inconspicuous. The phage attacks the strains of type 3a and 3b and some variants of type 1 Felix and Callow. Variants of this phage giving plaques with a clearer bottom, and variants having affinity for more subtypes of type 1 of Felix and Callow, were observed. Serologically these phages form a group by themselves, showing no cross reactions with other groups.

Phage II. This bacteriophage has been found in type 1 of Felix and Callow (e.g. strain 1 B76). The phage can easily be isolated by filtration of a 24 hours broth culture of a strain of this type. When a drop of this filtrate is tested on *S. Dublin* strain 65, a few to a hundred plaques appear. They are small and irregular and clear. The phage propagates easily on the strain: after a single passage, confluent lysis is seen.

Phage IV. This phage is found in a type Kampen [SCHOLTENS (18)] characterised by its lysogenic properties and peculiar phage reactions. Strains of this type produce phage I, and a second bacteriophage IV. This bacteriophage has two variants IVa and IVb. The former is quantitatively and qualitatively more virulent than the latter. Qualitatively it attacks all strains lysed by the second variant, and also the strains producing this phage itself. Nearly all strains attacked by phage IVa and not by phage IVb produce these two bacteriophages spontaneously. They constitute type "Kampen". It appeared that this type corresponded with type "Taunton" already found by FELIX and CALLOW and that the bacteriophages IVa and IVb have been included in their system as bacteriophages "Beccles" and "Taunton".

Phage VI. In filtrates of strains of various types bacteriophages are found which cannot be discerned with certainty by host range, plaque morphology and serology. These phages have plaques from pinpoint to $^1/_6$ mm of size with irregular form. In the larger plaques secondary growth may be seen. Serologically the bacteriophages contain at least one principal antigen in common. It should be borne in mind, that serological work in the field of bacteriophage is in the same primitive stage as the serology of bacteria before qualitative receptor analysis was introduced by WEIL and FELIX (20).

DESCRIPTION OF STRAINS AND TYPES.

The strains received from Dr FELIX as "type" strains, and strains isolated in the "Rijks Instituut voor de Volksgezondheid" were utilised. Only strains of special properties will be described here.

Type 1 of FELIX and CALLOW.

d–Tartrate positive strains were found in an epidemic following the consumption of meat of a hog slaughtered at a farm. In all cases the gastroenteritic form of Salmonellosis was observed. The strains reacted with all phage preparations, except the preparations IVa and IVb.

Type "Yersey".

These strains have been diagnosed with an adapted phage preparation, obtained from the preparation for type 1 of Felix and Callow. Strains indicated by this preparation were typed "Yersey" by Dr Felix.

Type Beccles (Meppel).

The specific preparation for type "Yersey" described above, attacked a group of strains, typed "Beccles" by Dr Felix, in an increased titre, but not in the critical test concentration. By means of a few passages a preparation was obtained attacking these strains in the same titre as the strains of type "Yersey". The greater part of the strains found in Holland, to be typed "Beccles" with the system of Felix and Callow, reacted with this preparation in the critical test concentration. They were found in association with strains of type "Yersey". The results are in accord with epidemiological data.

Type Beccles (Capelle a/d IJssel).

These strains reacted in an almost identical manner to phage preparations as the strains "Beccles" just mentioned, the adapted preparations included. However, they did not produce phage I, nor any other phage detectable with available test strains. A phenomenon, perhaps connected with this fact, was, that they were more sensitive to phage I than the strains mentioned above, giving confluent lysis, and plaques with strong concentrations of this phage (1000 × critical test concentration), or even "opaque lysis" in higher dilutions.

These concentrations did not at all attack strains of type "Beccles" producing phage I. In 1950 [Scholtens (18)] an outbreak caused by these strains was observed. Another outbreak affecting four members of a family caused by similar strains has been observed since. The source of the infection was not found.

The strains of type Yersey and of type Beccles (Meppel) produce phage I; the strains of type Beccles (Capelle a/d IJssel) do not produce this bacteriophage. In strains of these three groups bacteriophages producing small plaques are found attacking strains of other Salmonellas, i. e. strain 65 (R.I.V.) of *S. Dublin*, and strains 74 and 76 of the collection of the International Salmonella Centre (*S. gallinarum* and *S. london*).

Type "Midwoud".

The characteristics of this type (included in type Beccles by Dr FELIX) are described in this paper.

Types containing bacteriophage VI.

This bacteriophage is found:

a. In a group of strains of type 3aI of FELIX and CALLOW which are in every respect identical with the strain 3aI B624 of FELIX and CALLOW. They also contain the phage I.

b. In a group of strains — producing also the phages I and VI — characterised by phage reactions and lysogenic properties as will be described below. One of these strains was typed "Dundee" by Dr FELIX. As no strains of other description as to available phage reactions — to be typed "Dundee" with the system of FELIX and CALLOW — were found, these strains will be indicated "Dundee" only.

c. In a group of strains found in an enormous outbreak caused by infected meat in the village of Waalwijk. These strains were typed B.A.O.R. by FELIX. They are however, probably a subgroup of this type, for FELIX and CALLOW describe that type B.A.O.R. secretes phage "Beccles". The strains from Waalwijk, however, produce phage VI only. They will be indicated by "B.A.O.R. (Waalwijk)" in this paper. The type was first met with and described by NICOLLE and D'YVES HAMON (15).

d. In strains, typed "3a, variation 2" by Dr FELIX. These strains were observed in Holland only in an epidemic in Eindhoven, where the cooling water in a dairy factory had been contaminated by sewage water. In this epidemic types 3aI (B624) and 1 FELIX and CALLOW were also found.

BACTERIOPHAGES APPEARING IN MIXED CULTURES.

A mixed culture was made in broth with the strains *S. paratyphi* B 5671 (type "Dundee") and *S. paratyphi* B 1180 (type "Midwoud"). After incubating 24 hours the phages VI and I — also produced by the strains separately — were found. In the first few days the number of bacteriophage corpuscles was strongly reduced. After five days' incubation, however, the phage content of the mixed culture appeared to have increased, and, moreover to have changed in properties.

TABLE 1.

Survey of strains and types and bacteriophages isolated from them.

Type according to Felix and Callow	Strains	Type as defined by lysogenicity and phage reactions	Host range of bacteriophages										Bacteriophages characteristic for types indicated
			obtained from single strains						obtained from mixed cultures				
			I	VII	II	IVa	IVb	VI	Dundee / Midwoud		1 Felix and Callow / Midwoud		
1	B 76		—	cl	—	2+m	—	cl	cl	cl	cl	3 + m	II
2	B 228		—	—	—	2+m	—	cl	cl	cl	cl	3 + m	VII
3a	B 62		cl	cl	—	cl	cl	cl	cl	cl	cl	2 + m	
3aI	B 1305	Schiedam	—	cl	—	—	—	cl	cl	cl	cl	—	I
3aI	B 624	Leeuwarden	—	cl	—	—	—	—	cl	cl	cl	—	I, VI
3b	B 656		cl	cl	—	cl	cl	cl	—	cl	cl	3 + m	
Dundee	5671		—	cl	—	—	—	—	—	cl	cl	—	I, VI
Yersey	10375		—	cl	—	cl	cl	cl	cl	cl	cl	3 + m	I
Beccles	12468	Meppel	—	cl	—	cl	cl	cl	cl	cl	cl	3 + m	I
Beccles	6450	Capelle a/d IJssel	—	cl	—	cl	cl	cl	cl	cl	cl	3 + m	—
Beccles	1180	Midwoud	—	cl	—	cl	cl	cl	cl	—	—	cl	I
B.A.O.R.	9501	Waalwijk	—	cl	—	—	—	—	cl	—	—	cl	VI
Taunton	16356	Kampen	—	cl	—	cl	—	—	cl	cl	cl	± m	I, IV
S. Dublin			—	cl	cl	2+m	—	cl	cl	cl	cl	cl	

cl = confluent lysis.
±, 1+, 2+, 3+, increasing numbers of separate plaques.
m = minute, only visible with a handlens.

This table is incomplete; the bacteriophages found in the types 3a and 3b by Felix and Callow (9) and by Nicolle and d'Yves Hamon (15) are not indicated.

A filtrate of the week-old mixed culture produced plaques on the strains *S. paratyphi* B 5671 (Dundee) and *S. paratyphi* B 3aI B624. These strains were neither attacked by phage I nor by phage VI. The mixed cultures appeared now to contain a mixture of phages. As to host range two different sorts of phages were found. Phages possessing the first of these host ranges attacked all strains of *S. paratyphi* B except the strains of types Dundee and 3b (strain B656), phages possessing the other host range attacked in the critical test concentration all strains, except the strains of type "Midwoud" and "B.A.O.R. (Waalwijk)" ¹) (table I).

Also phages could be isolated from these mixed cultures by propagation on the strain *S. Dublin* 65.

With this strain preparations with high titre (critical test concentration $1/10^3$) could easily be obtained (these preparations proved of great value on producing antiphage sera).

It appeared that in these higher dilutions only the strain *S. Dublin* 65 (and other strains of *S. Dublin*) were attacked and the strains of *S. paratyphi* B were not. In strong concentrations (1000 × critical test concentrations for *S. Dublin* 65) strains of *S. paratyphi* B were attacked, and the two host ranges, described above, were found. It could be shown, that the preparations are not mixtures of different phages, for the propagation of the phage from single plaques on strain *S. Dublin* 65 (three times in succession) did not affect these phenomena.

The phages isolated and propagated on strains of *S. paratyphi* B did not show an exceptionally high titre for strain *S. Dublin* 65.

If, however, such phages were propagated on this strain, preparations were obtained similar to those obtained by a direct isolation on strain *S. Dublin* 65. They possessed an increased specific affinity for this strain (and for nine out of twelve other strains of *S. Dublin*). They attacked them exclusively in high dilutions, leaving the strains of *S. paratyphi* B untouched.

When stronger concentrations, however, were tested the host ranges for types of *S. paratyphi* B described above were still observed.

It may be assumed, that the preparations thus obtained are similar to the preparations isolated directly from the mixed cultures with strain *S. Dublin* 65.

Other phages produced by *S. paratyphi* B (e.g. phage VII found

---

¹) This host range corresponds with the host range of the preparation "Dundee" of the system of Felix and Callow.

TAB

Serological reactions of bacteriopha

BACTERIOPHAGES														
No.	Filtrate number	Obtained from (mixed) culture of strain(s) of type(s)		Isolated and propagated upon strain	Serum 27 anti phage VI (obtained from type Dundee)									
		strain(s)	type(s)		1/ 100	1/ 800	1/ 1600	1/ 3200	1/ 6400	1/ 12800	1/ 25600	1/ 100	1/ 40
VI		5671	Dundee	6450 Beccles Capelle a/d IJssel	—	—	1 pl.	+2	+3	+4	+4	+4	+
VI		3367	3aI	6450	—	—	+1	+2	+3	+4	+4	+4	+
VI		9501	B.A.O.R. (Waalwijk)	6450	—	—	1 pl.	±	±	+3	+4	+4	+
	553	4471 7121	Dundee Midwoud	65, *S. Dublin*	—	—	—	—	—	—	—	—	—
	548	4471 7121	Dundee Midwoud	65, *S. Dublin*	+3	+3	+3	+3	+3	+3	+3	—	—
	552	10375 7121	Yersey Midwoud	10375 Yersey	+4	+4	+4	+4	+4	+4	+4	—	—
	181	6773 1180	1 Felix and Callow Midwoud	1180 Midwoud	+4	+4	+4	+4	+4	+4	+4	—	—

Filtrates 553, 548, 552 and 181 are obtained with bacteriophages from mixed cultures.
+ 1, + 2, + 3, + 4 increasing number of plaques present.

with type 2 of Felix and Callow) also showed this property to develop a specific affinity for strains of *S. Dublin.*

SEROLOGY OF THE BACTERIOPHAGES FOUND.

Bacteriophages were isolated from the mixed cultures under examination and obtained "pure" by three successive subcultures from single plaques. Two serological groups were found. Firstly, a group of phages reacting in high dilutions of sera prepared with phage VI; and only in low dilutions (1/400) of the sera produced with phages of the second group (tabel II).

ained from (mixed) cultures.

TI BACTERIOPHAGE SERA

	erum 90 anti phage otained from mixed ıre Dundee + Midwoud filtrate 548					Serum 39 anti phage obtained from mixed culture Yersey + Midwoud filtrate 552						Serum 470 anti phage obtained from mixed culture type 1 FELIX and CALLOW + Midwoud filtrate 181						Control
	Dilution																	
	1/ 1600	1/ 3200	1/ 6400	/1 12800	1/ 25600	1/ 100	1/ 200	1/ 800	1/ 1600	1/ 3200	1/ 6400	1/ 100	1/ 800	1/ 1600	1/ 3200	1/ 6400	1/ 12800	
4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4
4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4
4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4
2	+3	+3	+3	+3	+3	+2	+3	+3	+3	+3	+3	+3	+3	+3	+3	+3	+3	+3
	—	—	—	±	+3	—	—	—	+1	+2	+3	—	—	±	±	+2	+3	+3
	—	—	—	+3	+4	—	—	—	—	+1	+2	—	—	—	—	—	+1	+4
	—	—	+3	+4	+4	—	—	—	—	—	+3	—	—	—	±	+1	+4	+4

The bacteriophages of the second group did not react at all with sera prepared with phage anti VI. A serum (serum 90) was prepared with one of these phages. This serum (tabel II) inactivated this second group of phages in a high titre (1/6400), but as mentioned above, the first group of phages only in low dilutions. The second group of bacteriophages reacted moreover with sera prepared with bacteriophages isolated from mixed cultures of type "Midwoud" and other types (these bacteriophages will be described below).

It appeared that both kinds of host range were present in both serological forms.

In this way four different types of bacteriophages were found. It was examined whether these types were constant. Three passages were made with each of these phages on the strains they were isolated with.

Now the preparations thus obtained were plated with this very strain and single plaques were obtained. Subcultures from eight single plaques showed serological properties and a host range similar to the original phage. However, some difficulty was encountered in the isolation of the serological variants. When the phage preparations obtained by heating the mixed cultures were plated on the sensitive strains [strains 1108 "Midwoud" and 6450 "Beccles (Capelle a/d IJssel)" being utilised preferably], plaques of different sizes were observed, small ones, and also larger ones in smaller numbers.

The subculture of the very small plaques yielded the phage preparations that did not react with the sera antiphage VI. When the larger plaques were subcultured preparations were generally obtained which reacted with none of the sera. When further passages were made, the sensitivity to serum antiphage VI appeared.

This may be explained in two different ways. It is possible, that mixtures were obtained, for the smaller plaques, sometimes hardly visible, far outnumber the larger ones. The second possibility is, however, that unstable forms play their part.

BACTERIOPHAGES FOUND IN MIXED CULTURES OF OTHER TYPES WITH STRAINS OF TYPE "MIDWOUD".

From mixed cultures of bacterial strains of the types 1 (Felix and Callow), "Yersey", "Beccles" (Meppel) and "Beccles (Capelle a/d IJssel)" with type "Midwoud" bacteriophages not found in the separate types were isolated. After a week's incubation the mixed cultures appeared to contain two different sorts of bacteriophages as regards host range.

One group attacked all strains except the strains of type "Midwoud" and "B.A.O.R. (Waalwijk)", the other ones preferably attacked these strains (the d-tartrate positive strains of type 1 Felix and Callow were attacked by both kinds of phages). These host ranges remained constant after many passages on the strains that were fully sensitive (table I).

Sera were prepared with bacteriophages from each of these mixed cultures. Though small differences in titre were observed it was not possible to find different serological phage variants in each of the mixed cultures, nor was it possible to distinguish the phages obtained from the mixed cultures of type "Midwoud" with different bacterial types.

The phages found did not react with the serum anti phage VI at all; they reacted strongly with serum 90, prepared with the other serological type of phages met with in the mixed cultures of strains of type "Midwoud" and type "Dundee".

The bacteriophages described are found with great regularity. They were found in all of 64 mixed cultures made with 8 strains of the type 1 of Felix and Callow, and 8 strains of the type "Midwoud" and in the mixed cultures of six strains of type "Beccles" (Meppel) with eight strains of type "Midwoud" 2, 2, 5, 6, 8 and 8 times respectively. Two strains of type "Midwoud" always produced the phenomenon.

EXPLANATION OF THE PHENOMENA DESCRIBED.

Considering the bacteriophages found in the mixed cultures of strains of type "Dundee" with strains of type "Midwoud" an explanation of the phenomenon may be given. It can be proved that one of these bacteriophages is formed out of elements derived from each of the two strains. This is the bacteriophage possessing the serology of the phage VI, and the host range comprehending all strains of *S. paratyphi* B except the strains "Midwoud" and "B.A.O.R. Waalwijk".

It is clear that the serology of bacteriophage VI derives from the strains of type "Dundee". This type produces a phage possessing this serology, whereas in pure or mixed cultures of strains of type "Midwoud", such a phage was never found, if no other strain, producing it was present.

The host range comprehending all types except types "Midwoud" and "B.A.O.R. (Waalwijk)" is derived from elements from the "Midwoud" strains. The appearance of phages possessing this host range depends on the presence of strains of type "Midwoud". They appear in the mixed cultures of strains of various types with the "Midwoud"type and are never found when these latter strains are absent.

Moreover, it is a generally observed phenomenon, that bacteriophages produced by a given type do not attack this very type

The bacteriophages discussed, attack type "Dundee" and not type "Midwoud".

Summing up we may say that bacteriophages arising in mixed cultures of these two types are formed by junction of elements from both strains. Now the two forms of hostrange and the two serological forms may give rise in different combination to four different types of bacteriophage. These four types have all been found. Therefore, it may be assumed that they are formed by a recombination of genetic elements.

Considering the bacteriophages found in mixed cultures of strains of type "Midwoud" with strains of various other types, it may be noted that a smaller variety of bacteriophages is found. It is, however, tempting to explain the appearance of bacteriophages not found in each of the strains apart, in a similar way.

These bacteriophages may be formed for the greater part out of elements derived from the strain of type "Midwoud", admixed, however, with elements derived from the other strains, whereby virulence is conveyed to the whole.

Further research will, however, show whether they are to be considered as "adaptations" of bacteriophages present in the strains under examination [Felix and Callow (5) showed in type 1 a bacteriophage attacking strains of type B.A.O.R.].

No properties of phage I are found back in the phages produced by recombination. Therefore, strains of type "Midwoud" probably contain elements of a second phage.

EXAMINATION OF OTHER STRAINS CONTAINING THE PHAGE VI, AND FREQUENCY OF THE PHENOMENON.

Of other types also producing the bacteriophage VI the type 3aI (B 624) of Felix and Callow was examined first. Like the strains of type "Dundee" these strains contain the phages I and VI.

Five strains of type "Dundee" and three strains of type 3aI (B 624) were grown with eight strains of the type "Midwoud". The strains of type "Dundee" initiated the phenomenon under discussion with 8, 8, 8, 7 and 5 strains of type "Midwoud" respectively.

With the strains of type 3aI (B 624), in one instance only, the formation of bacteriophages by recombination was seen.

The strain 14390 of this type of *S. paratyphi* was observed to produce new phages with one out of the eight strains of type "Midwoud"; in a repeated experiment with the eight strains of type "Midwoud" it did not show the phenomenon. The host ranges of the phages found were similar to the host ranges of phages from the mixed culture of type "Midwoud" with type 1 (Felix and Callow) or with other types respectively. Two serological types were found. One type reacted not only with serum 27 anti phage VI, but also with serum 90. The phages of the other type were neutralised by serum 90 only. The two forms of host range were found in both serological types.

Though many experiments were performed with strains of type 3aI (B 624) no further occurrence of this phenomenon was observed. All strains of this type (and of type "Dundee") which arrived at the laboratory for phage typing purposes were examined, and grown in mixed culture with the strain *S. paratyphi* B 7121, type "Midwoud". None of the 35 strains received showed the formation of bacteriophages by recombination in presence of that strain. The strains came at least from five different foci.

Three freshly isolated strains of type "Dundee" all gave rise to new phages by recombination. They came from the same outbreak.

Strains of type "B.A.O.R. (Waalwijk)" did not produce phages by recombination when grown in mixed culture with strains of type "Midwoud". Many strains, all coming from the one large epidemic caused by this type in Holland, were examined.

EXAMINATION FOR THE APPEARANCE OF NEW BACTERIAL TYPES IN MIXED CULTURES.

As bacterial types may be characterised by the phages they produce, and as phages have been found here, which are not produced by the strains inoculated in the medium, it might be possible to observe the genesis of new bacterial "types" from the mixed culture. The mixed culture was plated after a week's incubation, and 20 colonies were subcultured. They were typed and examined for their lysogenicity. The appearance of rough characters in most of the subcultures made typing impossible, and no strains were found secreting the new phages described.

CHARACTERISATION OF TYPES BY PHAGE REACTIONS AND LYSOGENIC PROPERTIES.

In the foregoing pages a type "Midwoud" was mentioned and worked with. In tabel I it may be seen that these strains may very well be distinguished from other types by their phage reactions. All strains found in the Netherlands giving these particular reactions appeared to possess the particular lysogenic characters described. Therefore, the type "Midwoud" can be characterised by its phage reactions and its lysogenic properties.

The strains giving phage reactions with the phages from table I identical with those of strain *S. paratyphi* B 5671 all secreted phages I and VI, and possessed the lysogenic properties in mixed cultures with type "Midwoud" described. These strains are indicated as type "Dundee" because they all probably belong to this type, and no strains of other description belonging to type "Dundee" have been found.

It has been pointed out, that a system of phage types of *S. paratyphi* B can be drawn in which the "types" are characterised by their lysogenic properties and by their phage reactions [SCHOLTENS (18)].

The system of phage reactions is given by the phages formed by spontaneous lysogenicity. This is a natural system of "phage types". From the study of lysogenicity the interpretation of the phage reactions can be derived. As a bacteriophage found by spontaneous lysogenicity does not attack the type by which it is formed, great value must be attached to some negative reactions.

FELIX and CALLOW (9) included several of the preparations of table I in their system. As they treat them as ,,adaptations" — which they are not — only a practical significance can be attributed to their system. It may, however, be accepted for certain, that a correct theoretical insight will further practical results.

BACTERIOPHAGES FOUND WHEN STRAINS ARE GROWN IN FILTRATES OF OTHER STRAINS.

There is no doubt that the phenomena dealt with above are caused by a filterable agent (the bacteriophage) which goes from one strain to the other. It was, therefore examined what result could be obtained if in the experiments mentioned above one of the strains was replaced by the filtrate of its broth culture.

It appeared that only in the mixed cultures of strains of type 1 of FELIX and CALLOW and strains of the type "Midwoud", results similar to those described were still obtained when one of the strains was replaced by its filtrate. In the combination filtrate type 1 + strain type "Midwoud", as well as in the combination strain type 1 + filtrate type "Midwoud" phages of both hostranges and similar serology were found.

Here it may be pointed out that a weak phage (derived from type 1 of FELIX and CALLOW) is added to a strain (of type ,,Midwoud") and induces the strain to secrete a phage composed chiefly of elements derived from this strain.

From the strain of type 1 of FELIX and CALLOW a bacteriophage can be isolated and propagated upon strain *S. Dublin* 65 [SCHOLTENS (17, 18), NICOLLE and D'YVES HAMON (15)]. If the strains of type "Midwoud" are incubated during a week with such a bacteriophage preparation, propagated on strain *S. Dublin* 65, a mixture of bacteriophages is found. These bacteriophages, however, differ from the phages obtained when the strains of type Midwoud are incubated with the filtrate of strains of type 1 of FELIX and CALLOW. A remarkable feature of the host range of the first mentioned is that they do not attack strain *S. Dublin* 65 in the critical test concentration, whereas the latter bacteriophages all attack this strain in the critical test concentration.

The bacteriophages isolated and propagated upon the strain *S. Dublin* 65 are not identical with the elements active in the filtrate of strains of type 1 of FELIX and CALLOW. The latter filtrate may contain more than one phage; it is, however, more, probable that phage elements present in the filtrate have been changed by a passage on strain *S. Dublin* 65.

THEORETICAL CONSIDERATIONS.

DOERR (6) says: "If lysogenicity seems to belong to one of the species characteristics of bacteria this is more in favour of an endogenic than of an exogenic origin of transmissible lysines".

The relationship found between the biological entity indicated as "phage type" and the phages produced by lysogenicity shows that lysogenicity is a species characteristic of bacteria. This is an indispensable link in a chain of evidence for an endogenic origin of bacteriophage.

Summary.

It was shown that bacteriophages, generated in mixed cultures of strains of *S. paratyphi* B of different types are formed by recombination of elements from both strains. The characteristics of the bacteriophages found depend upon the "phage type" of the strains inoculated in the medium.

Types of *S. paratyphi* B can be characterised by a combination of phage reactions and lysogenic properties.

I am indebted to Dr A. Felix and Miss B. Callow for putting the type strains and preparations of their system at my disposal, and for the examination of the numerous strains mentioned in this paper.

Literature.

1. J. S. K. Boyd, J. Path. Bact. **62,** 501, 1950. – 2. J. Craigie and K. F. Brandon, J. Path. Bact. **43,** 233, 1936. – 3. J. Craigie, Canad. Publ. Health J. **33,** 41, 1942. – 4. J. Craigie and A. Felix, Lancet 1947, 823. – 5. J. Graigie and C. H. Yen, Canad. Publ. Health J. **29,** 448, 484, 1938. – 6. O. Doerr, Handbuch der Virusforschung p. 51, 1938. – 7. A. Felix and B. R. Callow, Brit. Med. J. **2,** 127, 1943. – 8. A. Felix and R. M. Pitt, Lancet 1947, 186. – 9. A. Felix and B. R. Callow, Lancet 1951, 10. – 10. R. T. Fisk, J. Inf. Dis. **71,** 153, 1942. – 11. R. T. Fisk and Olga E. Mordvin, Am. J. Hyg. **40,** 232, 1944. – 12. K. Lilleengen, Acta Path. et microbiol. Scand. **24,** 591, 1948. – 13. M. Lisbonne et L. Carrère, C. R. Soc. Biol. **86,** 569, 1922. – 14. R. Otto und H. Munter, Deutsche Med. Woch. **47,** 1579, 1921. – 15. P. Nicolle et d'Yves Hamon, Ann. Inst. Past. **80,** 479, 1951; **81,** 614, 1951. – 16. R. Th. Scholtens, J. Hyg. **36,** 452, 1936. – 17. R. Th. Scholtens, Antonie van Leeuwenhoek **16,** 245, 1950. – 18. R. Th. Scholtens, Antonie van Leeuwenhoek **16,** 256, 1950. – 19. V. Sertic et N. A. Boulgakov, C. R. Soc. Biol. **122,** 35, 1936. – 20. E. Weil und A. Felix, Wiener Klin. Woch. 1918, 986. – 21. G. S. Wilson and J. D. Atkinson, Lancet 1945, 647.

(From the Bacteriological Laboratory of Lever Brothers and Unilever N.V.,
Rotterdam).

ENRICHMENT AND ISOLATION OF *STREPTOCOCCUS CITROPHILUS* VAN BEYNUM ET PETTE

by

A. KNETEMAN

(Received April 21, 1952).

INTRODUCTION.

In 1936 VAN BEYNUM and PETTE (1) described a new strain of lactic acid bacteria isolated by them in 1932 from spontaneously soured raw milk. They described this bacterium as a diplococcus, catalase negative, acidifying milk within a short time, forming dextro rotary lactic acid and fermenting maltose and dextrin. According to the classification of ORLA JENSEN it therefore had to be looked upon as a strain of *Streptococcus lactis*.

It differed however from all known strains of *Streptococcus lactis* by its capacity to ferment citrate vigorously, forming from it carbonic acid, acetic acid, acetoin and diacetyl. In this respect it resembled *Betacoccus cremoris* and therefore VAN BEYNUM and PETTE express the opinion that "this organism combines properties of a mixed culture of lactic streptococci and aroma betacocci". On behalf of its citrate fermenting property VAN BEYNUM and PETTE gave it the name: "*Streptococcus citrophilus*".

The citrate fermentation by *Str. citrophilus* results in the quantitative formation of acetoin (in the absence of air) according to the equation:

$$2 \text{ citric acid} \rightarrow 2 \text{ acetic acid} + 4 \text{ carbonic acid} + 1 \text{ acetoin}$$

which feature opened the possibility for VAN BEYNUM and PETTE to study the citrate decomposition by lactic acid bacteria (2).

VAN BEYNUM and PETTE further state that cultures of *Str. citrophilus* in milk have a disagreeable taste and smell which it also imparts to starters to which it is added. The bacterium is therefore considered as of no use in starters.

According to van Beynum and Pette *Str. citrophilus* is only seldom found in nature. It was never found by them in starters and was only on few occasions isolated from raw market milk.

Two features of this bacterium are not mentioned by van Beynum and Pette namely the formation of large amounts of acetoin from milk sugar in the presence of air and the liability of *Str. citrophilus* to form variants.

The formation of acetoin from sugar will be dealt with in the discussion. The variation is of a special interest. Variants are formed extremely easily in the strains isolated by me. Several different variants have been isolated already and will be described later in a separate publication. A variant most easily formed is one lacking the capacity to ferment citrate. Sometimes this variant appears so rapidly that constant isolations of the citrate fermenting type from disseminations of the stock cultures were necessary to keep the original strain. The original citrate fermenting form was until now never formed back from the citrate negative variant.

As to the fermentation of citrate this was studied by van Beynum and Pette in concentrations up to 0.4% in milk. In testing my own strains on citrate fermentation it was found that these were capable of fermenting up to 7.5% citrate added to milk. The fermentation is so vigorous that it can be compared with the fermentation of sugar by bakers yeast.

Though *Str. citrophilus* is of no interest in dairy industry its peculiar features as regards citrate fermentation and variation together with its rare appearence in nature make it an interesting object for further studies and make it worthwhile to publish in the following a mode of enrichment and isolation.

Own Isolations.

Strains of lactic acid bacteria showing the same properties as the strain found by van Beynum and Pette have been isolated in our laboratory several times and the peculiar feature of citrate fermentation was recognized and used for the study of citrate decomposition at about the same time as van Beynum and Pette and with the same results.

The first isolated strain dated from December 1930 and was found in a young starter. In the following year some strains have been isolated occasionally, again from young starters. Their number in starters was however exceedingly small and though afterwards hundreds of starters have been examined it was not found in them any more. The reason for this absence in starters will be discussed later on and the reason why VAN BEYNUM and PETTE did not find *Str. citrophilus* in starters will then be clear.

Though the above mentioned features had not been described I considered my strains after reading the publication of VAN BEYNUM and PETTE as identical to those found by them in milk. Some months ago we received from Dr PETTE one of his own strains, for which I express here again my best thanks. This strain, on examination showed no difference with those isolated by myself and disseminations of it showed that the same variants as found by me were already present in it. There is therefore no longer any doubt that the strains isolated by VAN BEYNUM and PETTE and my own strains are really identical.

As by the time of the first isolations this bacterium was of special interest e.g. for the study of citrate decomposition it was of importance to dispose of excellent strains at any time. The difficulty to find them in nature, to recognize the colonies among hundreds of others in disseminations and to keep strains in good condition without variation (freeze drying is excellent in this respect, but was not yet practised at that time) made it extremely valuable to dispose of a good enrichment method.

TRIALS FOR ENRICHMENT.

A. The use of citratemilk for enrichment.

It goes without saying that the main feature of *Str. citrophilus*, the fermentation of citrate, was tried in the building up of an enrichment method.

Portions of milk to which increasing quantities of citrate, up to 10%, were added, were inoculated with a suitable raw material, incubated at different temperatures and after growth transferred in the same media. The result even after several transfers, was negative. Even if *Str. citrophilus* was added together with the raw material this bacterium could not be enriched in this way. A strong fermentation of citrate with gas formation took place which however was due to the enrichment of Betacocci especially of those types which ferment citrate to carbonic acid, acetic acid and ethanol and hardly any C_4 products, the types which prevail in bad starters.

B. Temperature.

A more accurate study of the pure strains of *Str. citrophilus* showed that the optimum temperature for growth in milk was not 20° C., the temperature used for cultivation of starters, but considerably higher. The optimum was found to be about 30° C. At 25° C. growth was already much better than at 20° C. Difference between 25° C. and 30° C. is small, 30° C. being slightly better. Over 30° C. growth was retarded, at 37° C. there was still some growth but at 45° C. no development could be obtained any more.

Enrichment in citrate milk at higher temperature (25-30-37° C.) was no success either. These cultures invariably led to the enrichment of Betacocci or other strains of no interest in this respect.

C. Enrichment in starters.

More successful was the study of the behaviour of *Str. citrophilus* in starters at different temperatures. As the optimum temperature for *Str. citrophilus* had been found to be considerably higher than that at which dairy starters are cultivated it was expected that this temperature (20° C.) was too low for *Str. citrophilus* to compete with the strains of lactic acid bacteria which form the population of starters.

For studying the behaviour of *Str. citrophilus* in starters mixtures of starters and *S. citrophilus* were transferred regularly in pasteurised skim milk at different temperatures and after each transfer the number of colonies of *Str. citrophilus* in disseminations were counted. The disseminations were made on special agar media on which colonies of *Str. citrophilus* can easily be recognized among the great number of other lactic acid bacteria developing from the starter used. This medium will be described later on.

At **20° C.** the percentage of colonies of *Str. citrophilus* in the disseminations was found to decrease after each transfer and after 5 to 6 transfers *Str. citrophilus* could not be found any more in them even if a considerable amount of a culture of *Str. citrophilus* in milk had been added at the first inoculation. Thus due to the fact that 20° C. is too far below the optimum temperature for *Str. citrophilus* this bacterium cannot compete at this temperature with the lactic acid bacteria which form the population of a starter the latter growing far more rapidly at this temperature. So it is clear that *Str. citrophilus* will never be found in dairy starters which have been prepared for instance from raw milk by repeated transfers in milk at 20° C.; the number of transfers necessary before such starters can be used for the souring of cream for butter making always leads to the elimination of *Str. citrophilus* from them. This is confirmed by the fact that VAN BEYNUM and PETTE never found *Str. citrophilus* in starters.

An exception on this rule is the following. If a factory has the custom to prepare its own starters in the laboratory *Str. citrophilus* may be found in small numbers in the first transfers.

In these first transfers competition between different strains of bacteria is smaller, souring is still irregular and often slow and the flora is not yet in equilibrium. It was from these first transfers that accidentally strains of *Str. citrophilus* have been isolated in our laboratory.

At **30° C.** things are different. Being the optimum for *Str. citrophilus*, this bacterium develops rapidly. For most strains of lactic acid bacteria present in starters 30° C. is above the optimum and the growth of these will be slower. I based these facts on the results of disseminations which showed that by repeated transfers in milk of a starter to which *Str. citrophilus* had been added the relative number of colonies of *Str. citrophilus* found in these disseminations increased gradually with the number of transfers, while the relative number of the colonies of ordinary lactic acid bacteria decreased. Consequently enrichment of *Str. citrophilus* in starters at 30° C. seemed possible. As however *Str. citrophilus* will be very sparingly present in natural raw materials the question arises whether enrichment would take place if only a single cell of *Str. citrophilus* was added to a starter.

It turned out that this was really the case. For the experiment a series of bottles of pasteurised skim milk was inoculated with 1% of a starter which I knew to be free from *Str. citrophilus*. Some of the bottles were considered as blancs while the others got dilutions containing about 4 cells of *Str. citrophilus* each. The whole series was incubated at 30° C. and transferred daily. Disseminations showed that, except in the blancs, *Str. citrophilus* could be found back in increased number in the consecutive inoculations. The increase in number is slow. It sometimes takes up to 10 transfers fo find a considerable number of colonies of *Str. citrophilus*. This will depend on the starter used. Does this starter contain lactic acid bacteria which show better competition against *Str. citrophilus* then more transfers will be necessary.

Though enrichment was noticed in isolated cases at **37° C.** this was not a success. If the strains of the starter itself do not thrive well at 37° C. enrichment takes place. If however lactobacilli are present, which is usually the case in enrichment from raw materials, failure is certain as *Str. citrophilus* cannot compete with lactobacilli at this temperature.

Enrichment from raw materials.

According to the above the promising temperature for enrichment is 30° C. With raw materials as inoculum *Str. citrophilus* has to compete with all kinds of bacteria not present in starters and it was not sure that enrichment of *Str. citrophilus* would take place. Nevertheless enrichment is usually successful if raw material is used as inoculum. Enrichment fails however if growth of vigorous strains of lactobacilli takes place, the growth of these being so rapid and the acidity rising so high that competition is impossible for *Str. citrophilus.*

Not all strains of lactobacilli do however lead to mishap and the risk of getting the undesirable lactobacilli can be greatly reduced by:

a. Putting the first inoculation at 20° C.

b. Adding in the beginning a small amount of a dairy starter as used for ripening of cream.

Both methods will favour the growth of Streptococci in the further enrichment at 30° C.

As far as I know there is no raw material in which *Str. citrophilus* is present in considerable numbers. Raw milk is really the only substance from which it has been isolated occasionally. Most probably it is never present in raw milk in great number. Enrichment from raw milk was usually without success especially in winter when the total number of lactic acid bacteria in raw milk is relatively small.

An excellent material for enrichment is found to be the sludge which accumulates in dairy centrifuges used for separating the cream from unpasteurised raw milk. This sludge, a rubber like substance, contains all the dirt from the milk and is one of the most heavily infected materials one can imagine. It is a valuable material for isolations of all kinds of bacteria and has often been of great use to us for this purpose. As it may contain pathogens some care in handling is advisable.

The following calculation will give an idea of the value of this sludge for isolations. A dairy at Rotterdam gave the following informations: A centrifuge is emptied and cleaned after separating in it about 25 tons of raw milk and the sludge removed from it amounts to about 4 kg. This sludge forms a layer of about one inch thick on the inside wall of the separator. The milk is received once a day and is the mixture of the morning and evening milk.

If now we estimate the amount of milk per cow and per day to be 20 liters then the 4 kg sludge must be considered as to contain the dirt from the milk of more than a thousand cows and if we take the average number of cows per farm to be 40, which will presumably be too high, then the sludge represents the milk from 25 farms. Knowing further that the flora of the raw milk may differ from farm to farm and even from cow to cow then it will be clear that a better raw material containing the largest possible number of different bacterial strains, will hardly exist. As further the sludge is deposited in the centrifuge gradually in concentric films, a sample taken straight across the layer will represent an average sample of the same number of farms or cows. Fresh sludge can be easily obtained from any dairy every day.

Most probably *Str. citrophilus* is not very wide-spread in nature. Even in samples of sludge it is not always found, especially not in winter and the number in it is usually small. Perhaps it is only found on certain farms or even on certain cows.

Enrichment Method.

5 to 10 g of sludge taken straight across the layer, is thoroughly mixed in a mortar with 100 or 200 ml of milk, adding the milk in the beginning in very small quantities to get a thin homogeneous suspension. The suspension is filled in a bottle of about the double volume and incubated at 20° C. for two days. It is now thoroughly mixed and transferred in pasteurised skim milk. If the first bottle has not soured a drop of a starter is added at this first inoculation to provide sufficient lactic streptococci. In this case it is better to incubate this first inoculation again at 20° C. All further transfers are incubated at 30° C. For the first transfer a fairly large inoculum is used as *Str. citrophilus* may be present in only small number. The following transfers are carried out daily and with only a few drops of inoculum for which 60 ml bottles are large enough. After the 5th inoculation at 30° C. disseminations are made on special media, which allow an easy recognition of *Str. citrophilus*. If after 10 transfers *Str. citrophilus* is not found in disseminations this bacterium must be considered to be absent in the sample used. This is however seldom the case. We estimate that

in summer at least in 9 of 10 samples of sludge the enrichment has been successful.

Usually surface growth of *Oidium lactis* is observed in the enrichment. It may cause trouble by overgrowing large portions of the plates on which disseminations are made. Elimination of *O. lactis* from the enrichment cultures can be achieved by filling up the bottle containing the culture with boiling water till it overflows the neck taking care however not to disturb the curd. This is done just before transfering. After a few minutes the hot water is poured off again taking care to leave the curd undisturbed. After 3 to 4 transfers the curd will be solid enough to withstand this procedure and if carried out in one or in two consecutive transfers *Oidium* is usually eliminated.

Another way to eliminate *Oidium* is to inoculate in totally filled stoppered bottles. Due to gas formation in the first transfers this is more cumbersome.

ISOLATION MEDIUM.

As it is very difficult to pick out from disseminations on the usual media the colonies of *Str. citrophilus*, these being not essentially different from those of most other lactic acid bacteria, a special medium on which colonies of *Str. citrophilus* are very characteristic would be of great value.

By making use of the capacity of *Str. citrophilus* to decompose citric acid vigorously I succeeded in composing a medium on which colonies of *Str. citrophilus* can be easily distinguished from the colonies of other accompanying lactic acid bacteria.

The underlying principle is a way to detect the disappearance of citrate, added to the medium, by the activity of the *Str. citrophilus* colonies.

Several reactions of the ferric ion e.g. those with potassium thiocyanide, tannic acid, potassium ferrocyanide, can be inhibited by the addition of citrate through complex ion formation. Decomposition of the inhibiting citrate will result in a positive reaction of the ferric ion. Consequently if disseminations are made on a medium containing: 1. a ferric salt, 2. another substance which gives a visible reaction with ferric ions and 3. just sufficient citrate to inhibit this reaction, the decomposition of citrate by *Str. citrophilus* will result in ferric reaction in or under its colonies.

Two reactions were suitable viz., 1. The reaction with tannic acid. 2. The reaction with ferrocyanide. Both reactions are inhibited by citrate and the decomposition of citrate by *Str. citrophilus* in an agar medium results in violet colonies in the case of tannic acid and dark blue colonies (prussian blue) in the case of ferrocyanide.

The reaction with ferrocyanide gives by far the best results and will be described here. For a sensitive reaction special adjustment of the medium was necessary:

1. The concentrations of the ferric salt, ferrocyanide and citrate, had to be adjusted in such a way that the formation of prussian blue was just prevented by the citrate concentration to assure a rapid and intense coloration of the colonies of citrate decomposing bacteria.

2. In a medium in which the conditions under 1 are fulfilled a blue coloration is also obtained by the addition of an acid. The formation of lactic acid by lactic acid bacteria must therefore be as much as possible suppressed.

These conditions have been fulfilled by using a rather poor medium with an initial fairly low pH and of low buffering capacity.

Composition of the medium.

The following solutions have to be prepared:

A. Acid whey.

One liter of skim milk is heated in a waterbath to 95° C. and while stirring 12.5 ml N lactic acid are added to precipitate the casein. The casein in this way (so called cooked curd) contains practically all calcium and phosphate thus leaving a whey with small buffering capacity. The whey is filtered through muslin while hot. It has a pH of about 5.0 and is slightly turbid. Clarification is not necessary.

B. Potassium ferrocyanide solution 10%.

C. Solution of:

ferric citrate	1 g
sodium citrate	1 g
water	40 g.

Dissolution is best carried out by boiling the ferric citrate together with the sodium citrate and little water e.g. 2—3 ml, until dissolved and then adding the rest of the water.

D. Sterile milk.

E. Water agar containing 1.5% agar-agar.

Sterilisation of A, B and C is not necessary, they are keepable when boiled in cotton plugged flasks.

Preparing of plates.

The medium for preparing plates consisted of two parts which are mixed after being cooled to ± 40° C.

Part I: 50 ml water agar
1 ml solution C (ferric citrate—sodium citrate).
Part II: 40 ml acid whey A
10 ml sterile skim milk
1 ml potassium ferrocyanide B.

Part I and II both cooled to ± 40° C. are mixed and the mixture poured in Petri dishes. Part II is prepared at room temperature to prevent curdling of the milk.

Remarks.

a. Plates are not made too thick. A thin plate shows sooner the desired reaction.
b. A temperature considerably higher than 40° C. for mixing the medium will through the acid reaction damage the agar and will result in too soft plates and in coagulation of the milk added.
c. Freshly prepared plates are rather soft. It is therefore advisable to put them before inoculation in a cool place of about 10—15° C. for a quarter of an hour.
d. It is not advisable to increase the agar content. Growth is better and more characteristic on a medium with low agar concentration.
e. Keeping the plates for a long time in daylight imparts them a blue colour. The reason for this is not clear, probably by oxidation of citrate. It is advised to keep them in the dark.
f. The addition of the small quantity of sterile milk is necessary for better growth. This favourable influence of milk is generally observed in lactic acid bacteria.
g. Plates are inoculated with e.g. 1/10.000, 1/100.000 and 1/1.000.000 ml of an enrichment culture.
h. The surface of the plates being usually rather moist the dishes are put in the incubator upside down with in the lid a piece of thin cardboard on which a drop of glycerol is spread for the absorption of the excess of water. The next day the agar surface is dry and the glycerol paper is removed.
It is not advisable to sterilise the cardboard before use, as by sterilisation of cardboard volatile poisonous substances may be formed, most probably vanilin, resulting in the absence of growth above the cardboard.
k. The plates are incubated at 30° C. but it is advisable to leave them for the first 24 hours in the room until the agar surface is dry. A moist surface would give growth in smears instead of in separate colonies. Plates can also advantageously be dried in the above mentioned way before inoculation.

After 2 days at 30°C. blue coloration of *Str. citrophilus* colonies will already be visible and after about 5 days at 30°C. they can be easily recognized. After about 5 days: blueish black, flat, larger than those of other lactic acid bacteria, about twice the size. The appearance is dull, without lustre and wrinkled radially if observed through a magnifying glass. There is no or only slight coloration of the surrounding medium. Fig. 1 gives the exact image of typical colonies.

Colonies of other lactic acid bacteria: Smaller in size, glossy and more convex, colourless to white, later on often with small blue centre. Fig. 2 shows part of a culture plate on which the different colonies in an enrichment culture can be easily distinguished. Fig. 3 shows part of such a plate.

Strains of Betacocci will also develop on these plates. Decomposing citrate they will also have a blue colour. Growth of these bacteria is however rather poor on the plates. The colour of their colonies is less intense and often more violet. They can easily be distinguished from strains of *Str. citrophilus.*

Test on citrate fermentation.

If pure strains have been obtained by dissemination of blue colonies on the same medium these strains have to be tested on citrate fermentation. They are brought in sterile milk and incubated at 30° C. When soured 2.5% sodium citrate is added as a sterile concentrated solution and the mixture again incubated at 30° C. In the case of *Str. citrophilus* a strong gas formation takes place usually already after some hours or at all events the next morning. The liberation of the gas is best observed when swirling the bottle round. Considerable amounts of acetoin must be formed and can be indicated in the usual ways.

DISCUSSION.

The decomposition of citrate by *Str. citrophilus* leads to the quantitative formation of one mol acetoin from two mol citric acid. This is only true in the absence of air.

In the presence of oxygen from the air two other reactions take place simultaneously:

1. Formation of diacetyl from citrate.
2. Formation of acetoin (and diacetyl) from lactose.

In both reactions oxygen from the air serves as hydrogen acceptor and is reduced to hydrogen peroxide. The first reaction, diacetyl formation is not uncommon. The second, however, though also observed in several other lactic acid bacteria, is more interesting as large amounts of acetoin may be formed from sugar by *Str. citrophilus* under special conditions. Aeration is here necessary to provide sufficient oxygen. However the amount of hydrogen peroxide formed increases at the same time and may reach such a high concentration that it causes the death of the culture. It is possible to demonstrate the presence of hydrogen peroxide in the medium with the usual reagents as e.g. acidified potassium iodide-starch solution. A better way is to make use of manganese dioxide culture plates as described in a previous publication (3). On such plates the formation of H_2O_2 results in the dissolution of the MnO_2 round the colonies thus yielding transparent fields on the black background.

All streptococci present in starters produce more or less H_2O_2 and give these transparent fields, except many strains of Betacocci occurring in sour milk which do not form fields.

The fields produced by *Str. citrophilus* are, however, by far the largest. Fig. 5 shows a dissemination on a manganese dioxide plate of a starter in which *Str. citrophilus* is present. The photo is taken with transmittant light and shows the transparent fields formed. Fig. 6 shows the H_2O_2 formation of a pure culture of *Str. citrophilus*.

The increase in H_2O_2 in an aerated culture will gradually bring acetoin formation to a stop. If, however, catalase in sufficient quantity is added to decompose the H_2O_2, acetoin formation continues and considerable amounts can be demonstrated. The addition of adequate hydrogen acceptors also leads to increased acetoin formation by *Str. citrophilus*.

VARIANTS.

Two of the variants will be mentioned here. The first lacks the capacity to ferment citrate. It is easily found in disseminations on the so called "prussian blue plates" described on which it appears as white colonies instead of blue ones. They have so to say changed into ordinary lactic acid streptococci from which they cannot be distinguished any more.

The second variant possesses still the citrate decomposing capacity; its colonies, however, on prussian blue plates are totally different from those of the original strain. They are usually smaller in size, not flat as the original, but strongly inflated and glossy instead of wrinkled. Figs. 1 and 4 show the two different forms of colonies, while fig. 7 shows the different variants in a dissemination of a pure culture strain.

OCCURRENCE IN NATURE AND VARIATION.

The two facts: the rare occurrence of *Str. citrophilus* in nature and the extreme rate of variation into the non-citrate fermenting form makes it puzzling why *Str. citrophilus* in the original form can still be found in nature and why it does not get alltogether lost. Would there be some natural conditions which are not known as yet and which prevent variation? Or is it possible that somewhere under special conditions the original form is formed back again from the variant? Neither in our media nor in milk have we ever noticed this back formation.

Neither can the rarity of occurrence be understood and it would be very interesting to learn of any natural habitat where it develops abundantly under special conditions without variations.

It might e.g. be that certain conditions in stables favour the growth of *Str. citrophilus*. At all events I believe that there will be some places where it can be found in great number. The enrichment method described in this paper could possibly be an adequate way to solve this problem.

It will further be interesting to know whether this bacterium notwithstanding its rare occurrence will be found also in other countries. The enrichment method described will also in this case be of value to find the answer.

Summary.

Str. citrophilus described by VAN BEYNUM and PETTE in 1936 is one of the most interesting lactic acid bacteria. Its main features are:

1. Its vigorous citrate fermentation in which acetoin and diacetyl are formed.
2. The formation of much acetoin (and diacetyl) from sugar.
3. Its liability to variation.

A way for enrichment and isolation has been described. The enrichment is based on competition of *Str. citrophilus* with other lactic acid streptococci in starters at different temperatures. At 30° C. this competition leads to the relative increase in number of *Str. citrophilus* in consecutive transfers.

The isolation is based on a colour reaction by *Str. citrophilus* on a special agar medium. This medium contains a ferric salt, potassium ferrocyanide and citrate the amounts of which are so

regulated that the formation of prussian blue is just inhibited by the citrate concentration. Decomposition of the citrate by *Str. citrophilus* results in the formation of prussian blue in the colonies of this bacterium imparting them a deep blue colour by which they are recognized among others.

Str. citrophilus forms different variants very easily some of which have been described.

References.

1. J. van Beynum en J. W. Pette, Verslagen Landbouwk. Onderzoekingen **42,** 360, 1936. – 2. J. van Beynum en J. W. Pette, Idem **44,** 207, 1938. – 3. A. Kneteman, Antonie van Leeuwenhoek **13,** 55, 1947.

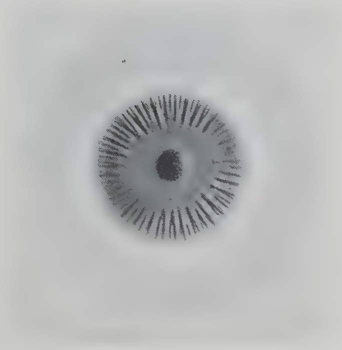


Fig. 1. Colony of *Str. citrophilus* on prussian blue agar. Original strain. Colony about 1 week old. 10 ×.

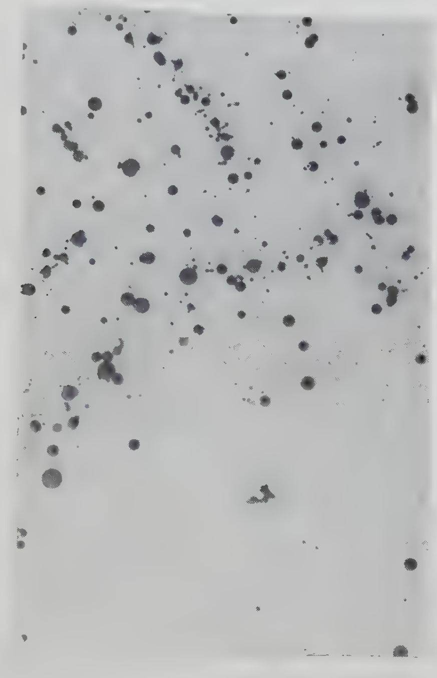


Fig. 2. Dissemination on prussian blue agar of an enrichment culture. 1.7 ×.

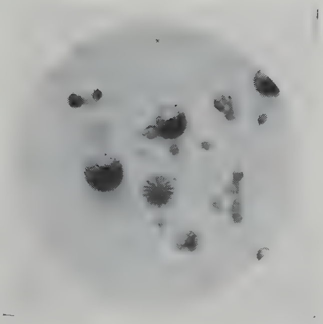


Fig. 3. Part of a prussian blue agar with dissemination of enrichment culture after 8 inoculations. Plate incubated 5 days on 30° C. 8 ×.

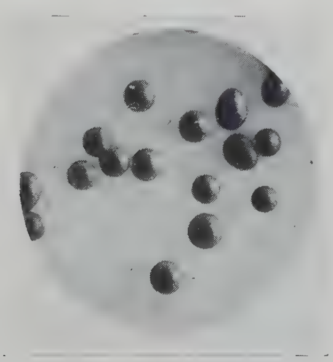


Fig. 4. Colonies of variant of *Str. citrophilus* on prussian blue agar, glossy inflated type. 10 ×.

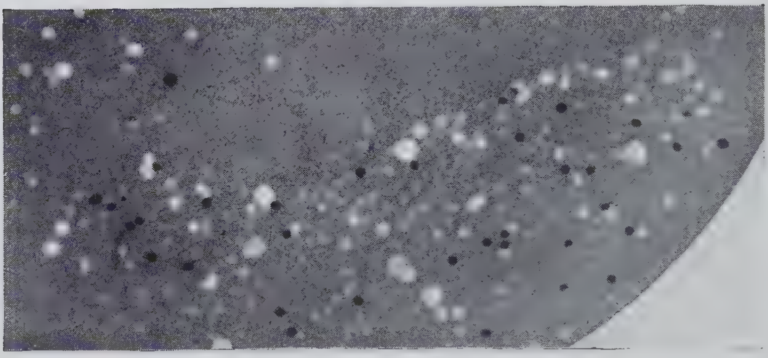


Fig. 5. Dissemination of a starter on manganese dioxide agar. 1.1 ×.

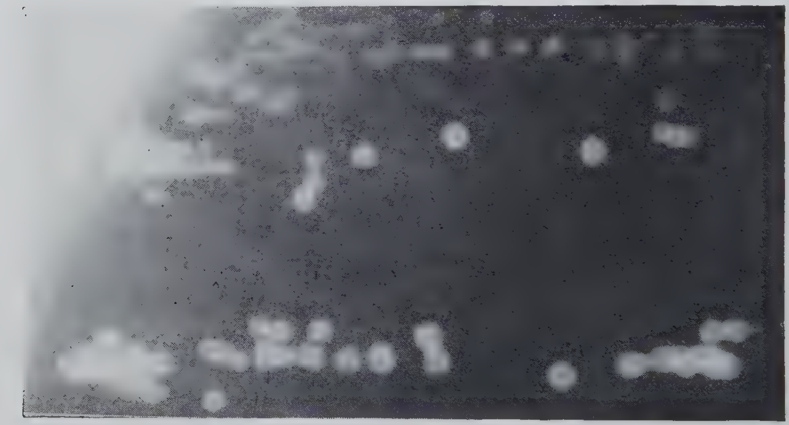


Fig. 6. Dissemination of a pure strain of *Str. citrophilus* on manganese dioxide agar. 1.8 ×.

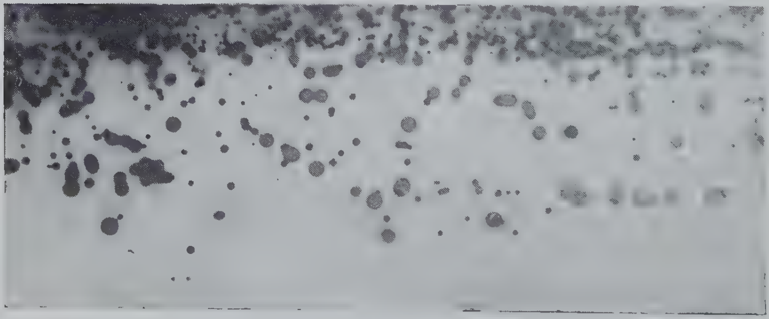


Fig. 7. Dissemination of a strain of *Str. citrophilus* on prussian blue agar showing different variants. After 7 days cultivation on 30° C. 1.7 ×.

(Laboratorium voor de Gezondheidsleer, Universiteit van Amsterdam).

NICOLAU STAIN FOR SMALLPOX DIAGNOSIS

by

F. DEKKING

(Received May 8, 1952).

In 1939 Nicolau (2) published a simple stain for purified elementary bodies which is eminently suited for the staining of smears from suspected cases of smallpox. Smears from lesions are made on a clean slide, fixed by heat or methylalcohol and stained for 10 minutes under gentle heating with the following stain: Isamine blue Gruebler 1 g, carbolic acid 3 g, ethanol 10 ml, distilled water 100 ml. The stain is prepared and used in the same way as the carbol-fuchsin stain.

Its advantages even over the relatively simple Gutstein stain are: 1. no preliminary treatment of the smear is necessary; 2. the stain is simple, keeps indefinitely and never gives precipitates; 3. the method is fool-proof: even very thick smears with much protein matter show clear cut and well-stained elementary bodies; and some slides, accidently left on an electric hot plate and completely dry when found gave clear and useful preparations after simple washing with destilled water.

Summary.

The Nicolau stain deserves to replace the Gutstein stain as a routine method for smallpox diagnosis; it is simple, effective and fool proof.

Literature.

1. Stains for smallpox diagnosis: C. E. van Rooyen and A. Rhodes, Virus diseases of Man 1948, p. 296. – 2. S. Nicolau, C. R. Soc. Biol. **130,** 993, 1939.

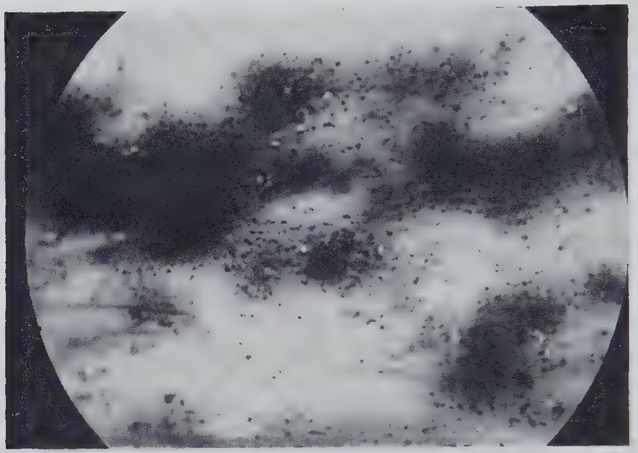


Fig. 1.

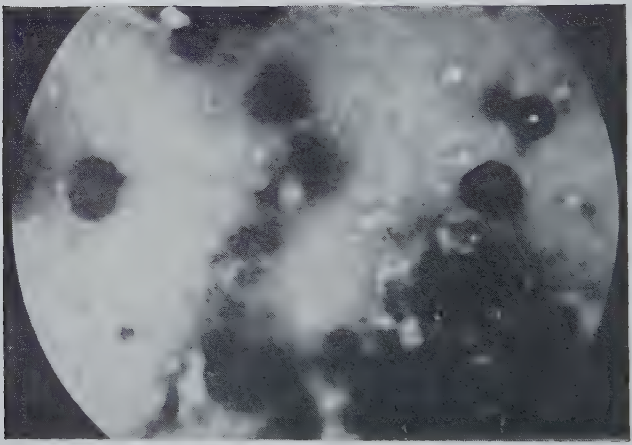


Fig. 2.

Microfotographs from the thick parts of two very unsatisfactory smears for smallpox diagnosis. Fig. 1 smallpox vesicle. Fig. 2 varicella vesicle. Nicolau stain. Fixative methanol. 1000 ×.

(Aus dem Institut für landw. Bakteriologie und Gärungsbiologie, Eidg. Techn. Hochschule, Zürich).

UNTERSUCHUNGEN ÜBER DIE PHYSIOLOGIE DER WEINHEFEN. III. MITTEILUNG.

UEBER DEN WUCHSSTOFFBEDARF EINER AUXO-HETEROTROPHEN, SCHWEIZERISCHEN KULTURWEINHEFE [1])

von

T. WIKÉN und **O. RICHARD**

(Eingegangen am 28. März 1952).

Kürzlich konnten wir zeigen, dass drei schweizerische Kulturweinhefen, die Rassen ,,Fendant'', ,,Herrliberg'' und ,,Salenegg'', den Charakter auxo-autotropher Organismen besitzen. Trotz ihrer ausgeprägten Auxo-Autotrophie reagiert die erste äusserst empfindlich auf sehr geringe Mengen (+)-Biotin und meso-Inosit, von denen bereits 0,000025 γ bzw. 2.5 γ in 1 ml Substrat die Vermehrung der Hefezellen fördern (3). Im Falle der ,,Herrliberg''- und ,,Salenegg''-Hefen übt (+)-Biotin weder auf die Geschwindigkeit der Zellvermehrung noch auf die maximale Ausbeute an Zellen einen Einfluss aus, während meso-Inosit eine bedeutende Stimulierung des Wachstums bewirkt (4). Auch ohne Zusatz von Wuchsstoffen zeigen aber die erwähnten drei Kulturweinhefen eine sehr rasche Vermehrung längs einer normalen S-Kurve. Die Hypothese, dass nur wilde Hefen imstande seien, in angemessener Zeit eine wuchsstofffreie synthetische Nährlösung zu assimilieren, den Kulturhefen aber dieses Vermögen fehle, hat somit keine allgemeine Gültigkeit. Anderseits weist, wie aus den folgenden Ausführungen hervorgeht, die schweizerische Kulturweinhefe ,,Dézaley'' eine ausgeprägte Auxo-Heterotrophie auf, indem ihre Zellen erst durch eine Kombination der drei Wuchsstoffe (+)-Biotin, meso-Inosit und (+)-Pantothensäure zur maximalen Vermehrung gebracht werden können. Bezüglich des Bedarfs an Vitaminen ist die Gruppe der Kulturweinhefen mithin durch eine sehr grosse Heterogenität gekennzeichnet.

---

[1]) II. Mitteilung: Antonie van Leeuwenhoek **18, 31, 1952.**

Abb. 1 zeigt das Aussehen der Riesenkolonie der ,,Dézaley''-Hefe auf Würzegelatine, und Abb. 2 die Sporenbildung in einer sehr alten Kolonie auf eingetrocknetem Substrat. Die Form der ,,Dézaley''-Zellen in ungehopfter Bierwürze, verdünntem Süsswein sowie den Nährlösungen A, B^1 und C (3) geht aus Abb. 3 hervor. In Saccharimetern nach Einhorn werden Fructose, Glucose und Saccharose unter Kohlendioxydbildung sehr rasch dissimiliert, während Maltose und Raffinose sowie besonders Galactose nur langsam angegriffen werden. Lactose wird nicht vergoren.

METHODIK.

In sämtlichen Versuchen kam die Methodik zur Verwendung, welche von uns in der I. Mitteilung in dieser Zeitschrift beschrieben wurde (3). Als Substrate dienten somit die Nährlösungen B^1 und C, welche beide Glucose als Kohlenstoff- und Energiequelle enthalten. Als Stickstoffquelle wird in der ersten Nährlösung Ammonsulfat und in der letzten vitaminfreies Caseinhydrolysat verwendet. Zur Gewinnung des Impfmaterials benützten wir in den meisten Versuchen das flüssige Substrat A, welches Malzextrakt neben Glucose, Ammonchlorid und anderen Mineralsalzen enthält. In einigen Fällen wurde das Impfmaterial auf Bierwürzeschrägagar gezüchtet. Als Mass des Wachstums wurde das Absorptionsvermögen der kräftig geschüttelten Kulturen für Licht der Wellenlänge 660 mμ gewählt, wobei die Messung der Lichtabsorption mit Hilfe eines Beckman-Quarzspektrophotometers in Corex-Küvetten mit einer Schichtdicke von 10 mm erfolgte. Die Spaltweite betrug 0,06 mm. Als Vergleichslösung (Durchlässigkeit = 100%) verwendeten wir die nicht geimpfte Nährlösung. In den Tabellen 1–12 sind die Durchlässigkeitswerte der Kulturen (%) als Mass des Wachstums angeführt.

Hier sei ferner erwähnt, dass sämtliche Versuche in Reagenzgläsern mit je 8 ml Nährlösung bei 20° C. durchgeführt wurden, wobei wir die Kulturen einmal täglich schüttelten.

ERGEBNISSE UND DISKUSSION.

Orientierende Versuche in Nährlösung C.

Im ersten Versuch wurde die Wirkung des (+)-Biotins (25 γ/1000

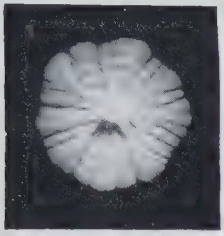


Abb. 1. Riesenkolonie der „Dézaley"-Hefe auf Würzegelatine (siehe STELLING-DEKKER (2), S. 30–31; auf 10° Bllg verdünnte Würze; 16% Gelatine). 6 Wochen alte Kultur. Natürliche Grösse.

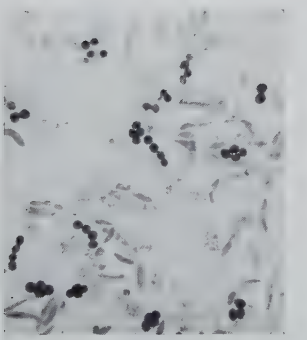


Abb. 2. Sporenbildung der „Dézaley"-Hefe in Riesenkolonie auf eingetrockneter Würzegelatine. Färbung mit Karbolfuchsin nach Behandlung mit Chromsäure. Vergr. 425 ×.

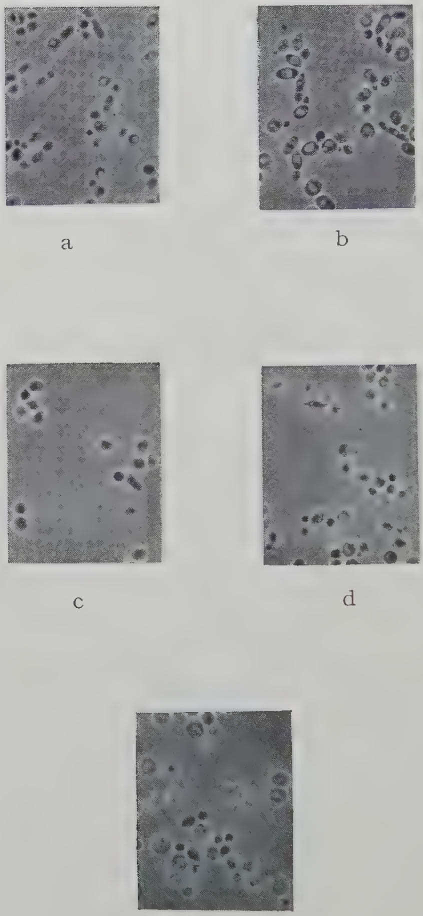


Abb. 3. Aussehen der ,,Dézaley''-Zellen in verschiedenen Nährlösungen. 48 Stunden alte Kulturen. Lebende Zellen. Phasenkontrast. Vergr. 300 ×.

a. Ungehopfte Bierwürze (8° Bllg).
b. Süsswein (Verd. 1 : 1).
c. Nährlösung A.
d. Nährlösung B[1].
e. Nährlösung C.

ml), des Adermins (0,5 mg/1000 ml), des Aneurins (0,5 mg/1000 ml), der (+)-Pantothensäure (2,5 mg Ca-Salz/1000 ml) sowie des meso-Inosits (25 mg/1000 ml) auf das Wachstum der ,,Dézaley''-Hefe in Nährlösung C geprüft. Als Impfmaterial dienten dabei gewaschene Zellen aus einer Kultur auf Bierwürzeschrägagar. Diese Kultur stammte von einer Hefekolonie auf einer Bierwürzeagarplatte, welche mit Zellen aus einer Reinkultur der Hefe in Weisswein geimpft worden war.

TABELLE 1.

Zeit Stunden	Kein Wuchsstoffzusatz	(+)-Biotin 25γ/1000 ml	Adermin 0,5 mg/1000 ml	Aneurin 0,5 mg/1000 ml	Pantothensäure 2,5 mg/1000 ml	meso-Inosit 25 mg/1000 ml
24	97,5	98,0	96,7	97,5	97,5	92,0
46	98,0	98,0	98,0	98,8	97,8	82,0
70	—	98,2	98,1	98,5	98,1	68,6
118	86,7	88,0	78,0	87,5	64,3	41,7
146	44,2	52,4	43,3	62,3	26,8	19,0
166	—	26,3	39,8	30,6	17,5	8,1
190	15,2	15,4	13,8	24,4	14,4	6,7

Impfkultur: Bierwürzeschrägagar, 48 Stunden alt.
Nährlösung: C. Impfung: 7.12.49. Einzelwerte.

Aus Tabelle 1 geht hervor, dass die so gewonnenen Zellen erst nach einer Induktionsperiode von etwa 120 Stunden im wuchsstofffreien Substrat C zur raschen Vermehrung fähig sind. (+)-Biotin, Adermin und Aneurin üben keine günstige Wirkung aus, während Pantothensäure und meso-Inosit insofern einen fördernden Einfluss haben, als das Wachstum bereits nach etwa 80 Stunden bzw. sofort nach der Impfung einsetzt.

Im zweiten Versuch wurde der Einfluss von (+)-Biotin und verschiedenen Kombinationen dieses Vitamins mit Adermin, Aneurin, (+)-Pantothensäure und meso-Inosit auf die Vermehrung der Hefe in Substrat C untersucht. Als Impfmaterial kamen Zellen von Bierwürzeschrägagar zur Verwendung. Der Schrägagar war mit einer Stammkultur in 10% Saccharoselösung geimpft worden.

Aus den in Tabelle 2 angeführten Ergebnissen ist ersichtlich, dass auch in diesem Versuch die ,,Dézaley''-Zellen erst nach einer Induktionszeit von etwa 120 Stunden die caseinhydrolysathaltige Nähr-

TABELLE 2.

Zeit Stunden	Kein Wuchsstoffzusatz	(+)-Biotin 25 γ/1000 ml	(+)-Biotin 25 γ/1000 ml						
							meso-Inosit 25 mg/1000 ml		
			Adermin 0,5 mg/1000 ml	Aneurin 0,5 mg/1000 ml	Pantothensäure 2,5 mg/1000 ml	meso-Inosit 25 mg/1000 ml	Adermin 0,5 mg/1000 ml	Aneurin 0,5 mg/1000 ml	Panthothensäure 2,5 mg/1000 ml
24	95,8	96,0	96,5	97,1	95,6	89,8	88,9	90,1	91,3
48	96,2	96,0	97,2	97,7	96,2	88,9	88,8	90,4	51,0
77	95,1	95,9	94,9	95,5	94,2	87,6	85,6	88,1	27,2
96	97,4	96,8	97,1	98,2	92,3	69,0	54,8	87,9	11,2
120	88,7	88,2	93,2	93,1	59,3	22,5	21,5	62,4	9,0
144	67,9	83,4	68,6	72,4	21,6	11,2	10,5	22,0	9,0
162	47,9	50,3	70,2	47,3	18,7	11,0	10,0	19,2	9,3

Impfkultur: Bierwürzeschrägagar, 72 Stunden alt.
Nährlösung: C. Impfung: 17.12.49. Einzelwerte.

TABELLE 3.

Zeit Stunden	Kein Wuchsstoffzusatz	(+)-Biotin 25 γ/1000 ml	Adermin 0,5 mg/1000 ml	Aneurin 0,5 mg/1000 ml	(+)-Biotin 25 γ/1000 ml		Pantothensäure 2,5 mg/1000 ml	
					Adermin 0,5 mg/1000 ml	Aneurin 0,5 mg/1000 ml	meso-Inosit 25 mg/1000 ml	(+)-Biotin 25 γ/1000 ml meso-Inosit 25 mg/1000 ml
24	93,4	93,3	93,8	93,3	94,3	95,1	87,1	81,8
48	91,5	94,0	94,7	95,0	94,3	95,3	63,0	39,8
72	94,3	94,8	94,1	94,9	95,3	95,9	54,9	17,1
96	92,8	94,3	95,0	95,0	95,1	96,1	38,0	10,1
120	92,0	95,5	95,8	96,1	96,2	96,5	36,2	9,2
168	87,3	94,0	94,0	94,3	86,1	93,3	21,0	9,8
192	85,8	91,9	93,9	94,1	77,3	89,0	19,0	9,4

Impfkultur: Bierwürzeschrägagar, 72 Stunden alt.
Nährlösung: C. Impfung: 9.1.50. Einzelwerte.

lösung ohne Zusatz von Wuchsstoffen zu assimilieren vermochten. Das (+)-Biotin allein sowie die Kombinationen des (+)-Biotins mit Adermin bzw. Aneurin zeigten keine wachstumsfördernde Wirkung. Dagegen wurde die Induktionsperiode in Anwesenheit der Gemische von (+)-Biotin und (+)-Pantothensäure bzw. meso-Inosit wesentlich abgekürzt. Im ersten Falle setzte das rasche Wachstum nach etwa 100 und im zweiten Falle bereits nach etwa 80 Stunden ein. In den letzten Kulturen begann die Zellvermehrung in der Tat sofort nach der Impfung, hörte aber nach 20-25 Stunden bei einer Durchlässigkeit von durchschnittlich 89% auf, und begann erst nach weiteren 50–55 Stunden wieder mit grosser Geschwindigkeit. Auf diese Erscheinung, welche mehr oder weniger ausgeprägt in den gleichzeitig mit (+)-Biotin und meso-Inosit versetzten Kulturen auftrat, kommen wir im folgenden zurück. Schnelle Vermehrung ohne Induktionszeit wurde nur in denjenigen Kulturen erhalten, welche mit einer Kombination von (+)-Biotin, (+)-Pantothensäure und meso-Inosit versetzt waren.

Die erwähnten Ergebnisse wurden durch die in einem dritten Versuch erhaltenen Daten bestätigt (Tabelle 3).

(+)-Biotin, Adermin und Aneurin, einzeln, sowie die Kombinationen des ersten Vitamins mit Adermin bzw. Aneurin übten keinen fördernden Einfluss auf das Wachstum der ,,Dézaley''-Hefe aus. Dagegen bewirkte das Gemisch von (+)-Pantothensäure und meso-Inosit eine kräftige Stimulierung der Zellvermehrung. Durch Zusatz von (+)-Biotin wurde dieser günstige Effekt wesentlich erhöht, indem das sofort nach der Impfung einsetzende Wachstum längs einer steil ansteigenden, S-förmigen Kurve verlief. Aus Tabelle 3 ist ferner ersichtlich, dass die ,,Dézaley''-Zellen (von Bierwürzeagar) in diesem Versuch ohne Zusatz von Wuchsstoffen nicht einmal nach einer Induktionsperiode von etwa 190 Stunden die Nährlösung C mit nennenswerter Geschwindigkeit zu assimilieren vermochten.

Der antagonistische Effekt des (+)-Biotins gegen meso-Inosit in Nährlösung C.

Es wurde bereits kurz erwähnt, dass bei gleichzeitigem Zusatz von (+)-Biotin und meso-Inosit eine gewisse Hemmung des fördernden Einflusses des letzten Wuchsstoffes auf die Vermehrung der ,,Dézaley''-Zellen im caseinhydrolysathaltigen Substrat C feststellbar ist. Dieser über verschiedene Zeitspannen dauernde antagonistische Effekt des (+)-Biotins gegen meso-Inosit konnte in zahlreichen Versuchen nachgewiesen werden. Durch die gleichen Versuche beabsichtigten wir, die Wirkung verschiedener Konzentrationen der beiden Wuchsstoffe sowie den Einfluss der Impfmenge abzuklären. Als Impfmaterial dienten dabei gewaschene Zellen aus dem malzextrakthaltigen Substrat A.

TABELLE 4.

Zeit Stunden	Kein Wuchsstoffzusatz	(+)-Biotin 25 γ/1000 ml	meso-Inosit mg/1000 ml			(+)-Biotin 25 γ/1000 ml meso-Inosit 25 mg/1000 ml
			0,25	2,5	25	
24	97,6	97,3	93,1	92,4	91,6	90,7
48	96,8	96,5	87,8	85,3	85,7	90,4
72	97,4	96,9	81,9	79,5	80,3	90,9
144	98,0	97,3	76,5	68,5	71,4	91,6
216	96,9	96,4	75,4	67,9	69,0	90,5
312	86,0	95,6	74,5	67,0	70,0	86,7

Impfkultur: Nährlösung A, 48 Stunden alt.
Nährlösung: C. Impfung: 28.8.50.
In den Reihen ohne Wuchsstoffzusatz bzw. 25 mg meso-Inosit/1000 ml 10 Parallelen, in übrigen Reihen 5 Parallelen.

Aus dem in Tabelle 4 angeführten Versuch geht hervor, dass meso-Inosit bereits in einer Konzentration von 0,25 mg je 1000 ml Nährlösung C wirksam ist. Diese Wirkung wird durch Erhöhung der Konzentration des Vitamins auf das zehn- bzw. hundertfache nur sehr wenig gesteigert. Im Vergleich zu den mit meso-Inosit allein versetzten Kulturen zeigen die Kulturen, welche ein Gemisch von 25 γ (+)-Biotin und 25 mg meso-Inosit je 1000 ml Substrat enthalten, eine deutliche Hemmung des Wachstums. Dieses hört nach einer Inkubationszeit von 20–25 Stunden bei einer Durchlässigkeit von durchschnittlich 90, 8% auf und setzt sich erst nach weiteren 190–195 Stunden fort.

Die erwähnten Ergebnisse werden durch die in Tabelle 5 wiedergegebenen Daten bestätigt.

Die Erhöhung der Konzentration des meso-Inosits von 0,25 auf 62,5 mg je 1000 ml Substrat, d.h. um das 250fache, hatte nur eine verhältnismässig geringe Steigerung der wachstumsfördernden

TABELLE 5.

Zeit Stunden	Kein Wuchsstoffzusatz	(+)-Biotin 25 γ/1000 ml	meso-Inosit mg/1000 ml			(+)-Biotin 25 γ/1000 ml		
						meso-Inosit mg/1000 ml		
			0,25	2,5	62,5	0,25	2,5	62,5
24	97,6	97,9	93,1	92,6	92,6	92,4	91,1	90,6
48	97,7	97,9	87,0	85,5	85,0	92,3	91,8	91,6
72	97,8	98,1	86,0	79,4	78,1	92,9	92,7	91,8
120	97,8	98,0	84,7	67,7	66,0	93,0	93,1	92,2
216	97,4	97,9	80,3	56,4	53,2	91,4	89,8	84,4
	96,6*	98,0*	77,6*	53,6*	52,1*	74,4*	53,0*	60,3*

Impfkultur: Nährlösung A, 24 Stunden alt. Nährlösung: C. Impfung: 6.9.50. In der Reihe ohne Wuchsstoffzusatz 15 Parallelen, in den Reihen mit 62,5 mg meso-Inosit/1000 ml bzw. 25 γ Biotin + 62,5 mg meso-Inosit/1000 ml 10 Parallelen, in übrigen Reihen 5 Parallelen.

*nach 144 Stunden Zusatz von 2,5 mg Pantothensäure/1000 ml.

Wirkung des Vitamins zur Folge. Das (+)-Biotin in einer Menge von 25 γ je 1000 ml Nährlösung C hemmte bei den drei geprüften Konzentrationen von meso-Inosit (0,25, 2,5 und 62,5 mg/1000 ml) durchgehend, indem das Wachstum nach 20–25 Stunden zum Stillstand kam. Die Durchlässigkeit der Kulturen blieb sodann bei durchschnittlich 92,7, 92,2 bzw. 91,6% für die Dauer von mindestens 100 Stunden unverändert. In diesem Versuch wurde ferner eine Anzahl der Kulturen nach 144 Stunden mit (+)-Pantothensäure versetzt. Der sehr kräftige wachstumsstimulierende Einfluss eines Gemisches von (+)-Biotin, (+)-Pantothensäure und meso-Inosit konnte dabei, wie aus Tabelle 5 ersichtlich ist, bestätigt werden (vgl. die Tabellen 2 und 3).

Aus den in Tabelle 6 wiedergegebenen Versuchsdaten geht hervor, dass (+)-Biotin nicht einmal in der Konzentration 100 γ /1000 ml Nährlösung C einen Wuchsstoffeffekt auf die ,,Dézaley''-Hefe ausübt. In Anwesenheit von 25 mg meso-Inosit je 1000 ml Substrat wurde durch Zusatz von 25 bzw. 50, 75 und 100 γ (+)-Biotin ein antagonistischer Effekt von der gleichen Grösse bewirkt. In allen vier Fällen hörte das Wachstum nach 20–25 Stunden auf, wobei die Durchlässigkeit der Kulturen bei durchschnittlich 91,6, 91,4, 91,6 bzw. 91,7% während der weiteren Dauer des Versuches unverändert blieb.

TABELLE 6.

Zeit Stunden	Kein Wuchsstoffzusatz	(+)-Biotin γ/1000 ml		meso-Inosit 25 mg/1000 ml	meso-Inosit 25 mg/1000 ml			
					(+)-Biotin γ/1000 ml			
		25	100		25	50	75	100
24	97,8	97,6	97,7	93,3	91,6	91,6	91,8	91,4
48	97,4	97,4	97,4	85,3	91,1	90,9	91,1	90,9
72	97,4	97,5	97,4	76,7	91,6	91,1	91,6	91,6
96	97,6	97,5	97,2	70,0	91,8	91,2	91,7	91,6
144	97,5	97,6	97,6	59,1	91,8	91,6	91,8	92,4
192	97,9	97,7	97,7	53,9	91,7	92,1	91,8	92,4

Impfkultur: Nährlösung A, 48 Stunden alt.
Nährlösung: C. Impfung: 26.9.50. 5 Parallelen.

TABELLE 7.

Zeit Stunden	Kein Wuchsstoffzusatz	(+)-Biotin γ/1000 ml		meso-Inosit 25 mg/1000 ml	meso-Inosit 25 mg/1000 ml			
					(+)-Biotin γ/1000 ml			
		0,0025	0,025		0,0025	0,025	0,25	2,5
24	99,0	99,0	99,0	98,0	97,7	97,7	97,6	97,7
48	98,4	98,6	98,4	96,8	97,7	97,3	97,4	97,2
72	98,7	98,7	98,7	96,4	96,4	97,4	97,4	97,7
120	98,7	99,0	98,8	96,0	96,5	97,4	96,8	97,7
168	98,5	98,9	98,8	90,4	92,7	96,3	96,0	95,5
240	98,8	98,8	98,8	78,0	79,5	88,2	88,4	85,8
288	98,6	98,5	98,6	69,0	70,4	65,3	47,9	69,8
384	98,7	98,5	98,4	49,4	48,4	37,5	15,0	13,6

Impfkultur: Nährlösung A, 48 Stunden alt.
Nährlösung: C. Impfung: 6.11.50. 5 Parallelen.

Der in Tabelle 7 angeführte Versuch wurde durchgeführt, um die kleinste gegen meso-Inosit antagonistisch wirkende (+)-Biotin-Konzentration zu bestimmen. Zufälligerweise kam dabei eine ausserordentlich kleine Impfmenge zur Verwendung. Die Durchlässigkeit der Kulturen betrug unmittelbar nach der Impfung etwa 99%. Die Ergebnisse sind deshalb nicht mit denjenigen der oben erwähnten

Versuche direkt vergleichbar. Immerhin konnte festgestellt werden, dass in Anwesenheit von 25 mg meso-Inosit je 1000 ml Nährlösung C bereits 0,025 γ (+)-Biotin in 1000 ml die wachstumsfördernde Wirkung des meso-Inosits während 240 Stunden deutlich zu hemmen vermögen. Anderseits wirkt (+)-Biotin in Mengen von 0,025–2,5 γ je 1000 ml Substrat bei weiterer Bebrütung der Kulturen steigernd

TABELLE 8.

Zeit Stunden	Kein Wuchsstoffzusatz	(+)-Biotin 25 γ/1000 ml	meso-Inosit 25 mg/1000 ml	(+)-Biotin 25 γ/1000 ml meso-Inosit 25 mg/1000 ml
	Schwache Impfung			
24	99,1	99,0	98,4	98,3
48	98,8	98,9	98,3	98,2
72	99,1	99,0	98,8	98,7
120	98,4	98,2	98,0	97,8
168	99,1	99,0	98,7	98,4
240	99,1	99,0	98,4	98,4
336	98,6	98,4	94,5	97,6
504	99,0	98,7	81,9	84,9
	Mittlere Impfung			
24	97,4	97,8	95,4	95,5
48	97,1	97,0	94,0	95,4
72	97,6	97,2	92,8	95,8
120	97,0	96,5	89,8	95,1
168	98,0	97,4	88,5	95,5
240	98,2	97,5	87,6	92,2
336	85,5	95,4	82,2	73,4
504	68,1	82,5	63,3	16,5
	Starke Impfung			
24	93,6	93,4	87,4	88,2
48	94,0	92,9	75,7	87,0
72	94,4	93,8	69,9	87,7
120	94,1	93,7	63,8	88,1
168	93,9	94,2	58,0	81,4
240	81,5	91,2	52,6	69,6
336	62,1	73,9	49,2	34,5
504	36,1	34,5	33,0	9,6
600	25,6	22,8	26,8	10,8

Impfkultur: Nährlösung A, 72 Stunden alt.
Nährlösung: C. Impfung: 27.11.50. 5 Parallelen.

auf den Wuchsstoffeffekt des meso-Inosits. Das (+)-Biotin allein ist im Konzentrationsgebiet 0,0025–0,025 γ/1000 ml unwirksam.

Um die Bedeutung der Impfmenge für die Interferenz zwischen (+)-Biotin und meso-Inosit zu untersuchen, wurde der in Tabelle 8 wiedergegebene Versuch ausgeführt. Dabei wurden die zur Impfung verwendeten Zellmengen so abgemessen, dass die Durchlässigkeit der Kulturen unmittelbar nach der Impfung 99 bzw. 97 und 94% betrug. Um nicht nur den zu Beginn der Zellvermehrung auftretenden Hemmungseffekt des (+)-Biotins, sondern auch die nach längerer Bebrütung eintretende wachstumsfördernde Wirkung dieses Vitamins in Anwesenheit von meso-Inosit zu beleuchten, dehnten wir die Versuchsdauer auf 500–600 Stunden aus.

Aus Tabelle 8 ergibt sich, dass die ,,Dézaley''-Hefe bei schwacher Impfung ($D \cong 99\%$) ohne Wuchsstoffzusatz oder mit Zusatz von (+)-Biotin allein nicht einmal nach etwa 500 Stunden in Nährlösung C zum Wachstum fähig war. Bei mittlerer ($D \cong 97\%$) und starker Impfung ($D \cong 94\%$) begann die Zellvermehrung in den erwähnten Kulturen nach etwa 250 bzw. 180 Stunden, wobei (+)-Biotin keinen fördernden Einfluss ausübte. Dagegen wurde durch meso-Inosit in allen drei Fällen eine Stimulierung des Wachstums bewirkt. Während diese bei starker und mittlerer Impfung bereits zu Beginn der Vermehrung eintrat, war sie bei schwacher Impfung erst nach etwa 250 Stunden sichtbar. Die Interferenz zwischen den beiden Vitaminen kam sowohl bei der starken, als auch bei der mittleren Impfung deutlich zum Vorschein. Die gleichzeitig mit (+)-Biotin und meso-Inosit versetzten Kulturen zeigten in beiden Fällen anfänglich Hemmung und später Stimulierung des Wachstums im Vergleich zu den Kulturen, welche nur meso-Inosit erhielten. Dabei betrug die Durchlässigkeit der Biotin-Inosit-Kulturen in der Hemmungsphase bei starker Impfung durchschnittlich 87,8 und bei mittlerer Impfung 95,5%.

Die Ergebnisse der speziellen Versuche mit (+)-Biotin und meso-Inosit in Nährlösung C (Tabellen 4–8) lassen sich folgendermassen zusammenfassen:

1. (+)-Biotin allein bewirkt im Konzentrationsgebiet 0,0025–100 γ/1000 ml Substrat über angemessene Zeiten keine Förderung der Vermehrung der ,,Dézaley''-Hefe (Versuchsdauer = 192–600 Stunden).

2. meso-Inosit allein übt in Mengen von 0,25 bis 62,5 mg je 1000 ml Nährlösung eine stimulierende Wirkung aus, indem bei

mittlerer bis starker Impfung (D = 97–94%) das Wachstum der Hefe ohne Induktionszeit einsetzt.

3. In gleichzeitiger Anwesenheit beider Vitamine lässt sich eine Interferenz nachweisen. Durch Zusatz von (+)-Biotin wird der Wuchsstoffeffekt des meso-Inosits in jungen Kulturen (Bebrütungszeit ≦ 240–312 Stunden) stark gehemmt, in alten Kulturen (Bebrütungszeit ≧ 240–312 Stunden) wesentlich gesteigert. In Gegenwart von 25 mg meso-Inosit je 1000 ml Substrat C übt (+)-Biotin bereits in der Konzentration 0,025 γ/1000 ml einen deutlichen antagonistischen Effekt aus. In quantitativer Hinsicht ist die Interferenz von der zur Impfung verwendeten Zellmenge abhängig.

Spezielle Versuche mit (+)-Pantothensäure sowie Kombinationen dieses Vitamins mit (+)-Biotin und meso-Inosit in Nährlösung C.

Aus den orientierenden Versuchen (Tabellen 1–3) ging hervor, dass (+)-Pantothensäure allein sowie Gemische dieses Wuchsstoffes mit (+)-Biotin und meso-Inosit eine wachstumsfördernde Wirkung auf die ,,Dézaley''-Hefe ausüben. Dagegen zeigten nur diejenigen

TABELLE 9.

Zeit Stunden	Kein Wuchsstoffzusatz	(+)-Biotin 25 γ/ 1000 ml	meso-Inosit 25 mg/ 1000 ml	Pantothensäure 2,5 mg/ 1000 ml	(+)-Biotin 25 γ/ 1000 ml meso-Inosit 25 mg/ 1000 ml	Pantothensäure 2,5 mg/1000 ml		
						(+)-Biotin 25 γ/ 1000 ml	meso-Inosit 25 mg/ 1000 ml	(+)-Biotin 25 γ/ 1000 ml meso-Inosit 25 mg/ 1000 ml
24	98,1	98,0	93,4	97,9	92,6	97,7	89,9	86,1
48	97,9	97,5	83,5	97,2	92,1	97,2	72,1	38,9
72	98,2	97,9	74,8	97,1	92,3	97,2	60,5	17,3
120	97,7	97,3	62,5	94,7	92,0	92,4	43,4	7,8
168	98,1	97,8	60,6	87,1	92,8	75,4	31,0	7,6
216	97,5	97,3	55,8	76,2	89,4	48,0	20,5	6,3
264	97,7	97,0	50,3	70,2	89,3	31,4	20,8	6,1
312	98,6	98,2	49,9	50,9	86,2	20,5	21,3	5,9

Impfkultur: Nährlösung A, 24 Stunden alt.
Nährlösung: C. Impfung: 13.9.50. 5 Parallelen.

TABELLE 10.

Zeit Stunden	Kein Wuchsstoffzusatz	(+)-Biotin 25 γ/ 1000 ml	Pantothensäure 2,5 mg/ 1000 ml	meso-Inosit 25 mg/ 1000 ml	(+)-Biotin 25 γ/1000 ml		Pantothensäure 2,5 mg/ 1000 ml meso-Inosit 25 mg/ 1000 ml
					Pantothensäure 2,5 mg/ 1000 ml	meso-Inosit 25 mg/ 1000 ml	
24	99,2	99,0	98,9	98,2	99,0	98,1	94,7
48	98,6	98,6	98,3	97,3	98,2	97,6	70,2
72	99,0	98,7	98,4	97,8	98,4	98,1	54,5
120	99,0	99,1	98,6	97,0	98,2	98,2	26,7
168	99,0	99,0	91,1	94,7	92,6	96,6	16,8
216	98,9	99,0	74,9	86,8	68,7	92,7	16,8
264	98,8	98,9	66,5	78,6	46,1	83,0	18,7
336	98,3	97,5	51,1	57,7	23,6	23,9	20,5
384	98,5	96,0	42,2	49,8	10,6	10,9	18,5
432	97,6	98,3	36,7	41,8	11,4	11,9	19,6
504	94,6	96,9	25,1	27,8	11,4	10,1	19,2
552	—	—	21,3	22,7	11,0	10,9	19,2
600	—	82,3	19,2	18,3	11,3	11,1	20,1
720	83,9	73,4	20,5	16,0	10,9	10,2	18,6
888	69,1	14,7	20,5	16,6	11,5	9,9	17,7

Impfkultur: Nährlösung A, 48 Stunden alt.
Nährlösung: C. Impfung: 6.11.50. 5 Parallelen.

Kulturen, welche alle drei Vitamine enthielten, maximale Zellvermehrung sofort nach der Impfung. Weitere Versuche wurden nun ausgeführt, teils um diesen Effekt zu bestätigen, teils um den Verlauf des Wachstums über längere Zeiten kennen zu lernen. Die Ergebnisse sind in den Tabellen 9 und 10 angeführt.

Tabelle 9 zeigt eindeutig, dass (+)-Pantothensäure gleich dem meso-Inosit als ein Wuchsstoff für die ,,Dézaley''-Hefe in Nährlösung C fungiert. Während aber bei der verwendeten Impfmenge die wachstumsfördernde Wirkung des meso-Inosits durch Zusatz von (+)-Biotin während der ganzen Versuchsdauer stark gehemmt wurde, trat in den gleichzeitig mit (+)-Pantothensäure und (+)-Biotin versetzten Kulturen kein antagonistischer Effekt ein. Es konnte im Gegenteil bereits nach etwa 75 Stunden ein steigernder Einfluss des (+)-Biotins auf den Wuchsstoffeffekt der (+)-Pantothensäure festgestellt werden. In den Kulturen, welche ein Gemisch von (+)-Pantothensäure und meso-Inosit enthielten, setzte die Zellvermehrung wie in den Inosit-Kulturen sofort nach der Impfung ein. Sie verlief sodann viel rascher und führte zu einer wesentlich höheren Ausbeute an Zellen als in den mit meso-Inosit bzw. (+)-Pantothensäure allein versetzten Kulturen. Optimale Wachstumsbedingungen lagen erst nach Zusatz einer Kombination von (+)-Biotin, (+)-Pantothensäure und meso-Inosit vor. Wie im zweiten orientierenden Versuch (Tabelle 2) fing dabei die Zellvermehrung ohne Induktionszeit an und verlief sodann längs einer steil ansteigenden, S-förmigen Kurve. Tabelle 9 bestätigt ferner, dass (+)-Biotin allein über angemessene Versuchszeiten (312 Stunden) in Nährlösung C unwirksam ist.

In einem weiteren Versuch, dessen Resultate in Tabelle 10 angeführt sind, konnten die oben erwähnten Befunde im Prinzip bestätigt werden. In diesem Versuch kam eine sehr kleine Impfmenge zur Verwendung (Durchlässigkeit der Kulturen sofort nach der Impfung $\leqq 99\%$). Es ist ersichtlich, dass in den Kulturen ohne Vitaminzusatz nur eine sehr unbedeutende Zellvermehrung erfolgte und zwar setzte diese erst nach etwa 450 Stunden ein. Ueber eine Versuchsdauer von etwa 500 Stunden hatte (+)-Biotin keinen Wuchsstoffeffekt. Dagegen war nach 600–700 Stunden eine wachstumsfördernde Wirkung durch dieses Vitamin feststellbar. Ein Zusatz von (+)-Pantothensäure bzw. meso-Inosit ermöglichte eine verhältnismässig rasche Zellvermehrung nach einer Induktionszeit von etwa 120 Stunden. Durch Zusatz von (+)-Biotin wurde der Wuchsstoffeffekt der Pantothensäure wesentlich gesteigert und derjenige des meso-Inosits anfänglich (Bebrütungszeit $\leqq 264$ Stunden) gehemmt, später kräftig gesteigert. In Anwesenheit des Gemisches von (+)-Pantothensäure und meso-Inosit wurde die caseinhydrolysathaltige Nährlösung ohne Induktionsperiode rasch assimiliert.

Versuche in Nährlösung B^1.

Es sei zuerst daran erinnert, dass die Nährlösung B^1 Ammoniumsulfat als einzige Stickstoffquelle enthält, während in Substrat C der für die Zellsubstanzsynthese nötige Stickstoff von dem bei der sauren Hydrolyse des Caseins entstehenden Aminosäuregemisch geliefert wird (3).

Aus Tabelle 11 geht hervor, dass die ,,Dézaley''-Hefe bei mittlerer Impfung (Durchlässigkeit der Kulturen unmittelbar nach der

TABELLE 11.

Zeit Stunden	Kein Wuchsstoffzusatz	(+)-Biotin 25 γ/1000 ml	meso-Inosit 25 mg/1000 ml	Pantothensäure 2,5 mg/1000 ml	(+)-Biotin 25 γ/1000 ml meso-Inosit 25 mg/1000 ml	Pantothensäure 2,5 mg/1000 ml		
						(+)-Biotin 25 γ/1000 ml	meso-Inosit 25 mg/1000 ml	(+)-Biotin 25 γ/1000 ml meso-Inosit 25 mg/1000 ml
24	96,5	96,5	95,3	96,5	91,3	96,4	95,7	89,3
48	95,9	96,5	90,8	95,8	82,1	96,6	91,0	66,2
72	94,4	95,2	85,3	94,4	76,9	96,1	85,5	47,1
120	91,7	96,4	76,8	91,9	71,0	96,2	76,9	25,2
168	87,7	94,6	72,6	88,8	68,4	96,0	72,8	15,2
216	84,0	80,3	69,3	84,2	65,8	89,1	68,2	15,3
264	80,5	50,8	66,7	81,3	53,8	69,1	64,2	15,9
312	78,6	44,5	65,2	79,9	34,4	48,2	61,8	15,5

Impfkultur: Nährlösung A, 24 Stunden alt.
Nährlösung: B^1. Impfung: 20.9.50. 5 Parallelen.

Impfung ≌ 97%) imstande ist, die wuchsstofffreie Nährlösung B^1 ohne Induktionszeit langsam zu assimilieren. Das (+)-Biotin wirkt anfänglich (Bebrütungszeit ≦ 168 Stunden) schwach hemmend, später aber stark stimulierend auf die Vermehrung der Hefe. Der meso-Inosit übt ohne Induktionsperiode einen wachstumsfördernden Einfluss aus, während die Zellteilung in den Kulturen mit (+)-Pantothensäure allein über die ganze Versuchszeit (312 Stunden) nicht grösser als in der Kontrollreihe ohne Zusatz von Wuchsstoffen ist. Durch Zusatz von (+)-Biotin wird die wachstumsstimulierende Wirkung des meso-Inosits gesteigert. Dieser günstige Effekt setzt bei der verwendeten Impfmenge sofort nach der Impfung ein, wird aber erst nach 216 Stunden besonders auffallend. Dagegen darf auf Grund der in Tabelle 11 angeführten Versuchsergebnisse der (+)-Pantothensäure kein solcher Effekt zugeschrieben werden. Eine ohne Induktionsperiode eintretende und sodann mit maximaler Geschwindigkeit verlaufende Zellvermehrung wird in Nährlösung B^1 erst erhalten, wenn die Kulturen mit einer Kombination der drei Wuchsstoffe (+)-Biotin, meso-Inosit und (+)-Pantothensäure versetzt werden.

TABELLE 12.

Zeit Stunden	Kein Wuchsstoffzusatz	(+)-Biotin 25 γ/1000 ml	Pantothensäure 2,5 mg/1000 ml	meso-Inosit 25 mg/1000 ml	(+)-Biotin 25 γ/1000 ml		Pantothensäure 2,5 mg/1000 ml meso-Inosit 25 mg/1000 ml
					Pantothensäure 2,5 mg/1000 ml	meso-Inosit 25 mg/1000 ml	
24	99,3	99,3	99,1	99,0	99,4	99,1	98,1
48	98,3	98,8	98,2	96,3	98,8	97,5	92,2
72	98,3	99,1	98,6	94,5	99,1	97,9	87,1
120	97,2	99,0	98,4	90,6	99,0	97,1	79,3
168	96,0	98,8	98,8	87,2	99,0	97,4	74,3
216	94,7	98,0	97,8	85,1	96,9	96,2	70,7
264	94,0	79,9	90,3	85,5	82,4	90,9	67,8
336	93,5	49,6	87,0	81,1	58,2	53,0	63,8
384	94,1	37,0	82,9	82,7	42,6	26,6	63,3
432	92,8	20,6	80,4	82,5	28,2	12,6	62,6
504	93,0	11,8	74,8	83,5	12,8	9,3	59,0
552	92,3	13,5	79,2	83,4	11,8	9,3	58,0
600	91,3	11,6	70,6	80,6	13,4	10,1	57,2
720	90,4	11,1	70,7	77,5	12,6	8,9	53,8
888	90,7	12,6	64,7	78,2	12,7	11,8	53,5

Impfkultur: Nährlösung A, 48 Stunden alt.
Nährlösung: B^1. Impfung: 6.11.50. 5 Parallelen.

Tabelle 12 zeigt die Ergebnisse eines weiteren Wachstumsversuches in Nährlösung B^1. Wir impften mit einer sehr kleinen Zellmenge (Durchlässigkeit der Kulturen sofort nach der Impfung $\cong$ 99,5%). Es ist ersichtlich, dass die „Dézaley"-Zellen auch bei dieser ausserordentlich schwachen Impfung fähig sind, das Substrat B^1 langsam zu assimilieren. Bezüglich des Einflusses von (+)-Biotin und meso-Inosit, einzeln oder gleichzeitig zugesetzt, wurden die in Tabelle 11 wiedergegebenen Resultate bestätigt. Somit übte das erste Vitamin anfangs (Bebrütungszeit $\leqq$ 216 Stunden) eine leichte Hemmung, später eine kräftige Stimulierung auf das Wachstum der Hefe aus, während der letzte Wuchsstoff eine Assimilation der Nährlösung sofort nach der Impfung ermöglichte. Dabei führte der Zusatz von (+)-Biotin praktisch zur maximalen Ausbeute an Hefezellen (D $\cong$ 12% nach etwa 500 Stunden). In den mit meso-Inosit versetzten Kulturen war dagegen die pro Volumeneinheit der Kulturflüssigkeit erreichte Zellenzahl selbst nach Bebrütung während fast 900 Stunden sehr gering (D $\leqq$ 78%). In Anwesenheit des (+)-Biotins wurde die wachstumsfördernde Wirkung des meso-Inosits während der ersten 264 Stunden gehemmt, nachher aber so kräftig gesteigert, dass die Geschwindigkeit der Zellvermehrung durchschnittlich grösser ist als in den Kulturen mit (+)-Biotin allein. In diesem Versuch zeigte ferner (+)-Pantothensäure einen Wuchsstoffeffekt, welcher unmittelbar nach der Impfung einsetzte. Wie im Falle des meso-Inosits wurde dabei nur eine verhältnismässig kleine Höchstausbeute an Hefezellen erhalten (D $\leqq$ 65%). Die Kulturen, welche sowohl (+)-Pantothensäure als auch meso-Inosit enthielten, zeigten anfangs grössere Wachstumsgeschwindigkeit als die Kulturen mit (+)-Pantothensäure allein. Jene Kulturen erreichten ferner eine höhere maximale Zellausbeute (D $\leqq$ 54%) als diese. Bei gleichzeitiger Gegenwart von (+)-Pantothensäure und (+)-Biotin verlief die Zellvermehrung wie in den Kulturen, welche mit dem letzten Wuchsstoff allein versetzt worden waren (D $\leqq$ 12,5 % nach etwa 500 Stunden).

Die Abhängigkeit des Wuchsstoffbedarfes der „Dézaley"-Hefe von der Zusammensetzung des Substrats.

Die in dieser Arbeit behandelten Versuchsergebnisse mit gewaschenem Impfmaterial aus Nährlösung A zeigen, dass zwischen dem Wuchsstoffbedarf der „Dézaley"-Zellen im caseinhydrolysathaltigen Substrat C und demjenigen in Substrat B^1, welches Ammonsulfat als Stickstoffquelle enthält, u.a. folgende Unterschiede bestehen:

1. Ohne Zusatz von Wuchsstoffen findet in Nährlösung C über angemessene Zeitspannen (je nach der Impfmenge 240–500 Stunden) keine Vermehrung der Hefe statt, während die wuchsstofffreie Lösung B^1 bereits nach 48–120 Stunden von den Zellen langsam assimiliert wird.

2. In Substrat C ist eine wachstumsfördernde Wirkung des (+)-Biotins erst nach 600–700 Stunden, in Substrat B^1 dagegen bereits nach 215–260 Stunden feststellbar (25 γ (+)-Biotin/1000 ml).

3. (+)-Pantothensäure übt in Lösung C bereits nach 75–150 Stunden einen günstigen Einfluss auf die Zellvermehrung aus, während in Nährlösung B^1 dieser Effekt erst nach 265 oder mehr Stunden einsetzt (2,5 mg Ca-Pantothenat/1000 ml).

4. In Nährlösung C lässt sich ein antagonistischer Effekt des (+)-Biotins gegen meso-Inosit nachweisen, indem in gleichzeitiger Anwesenheit beider Vitamine die wachstumsfördernde Wirkung des letzten Wuchsstoffes durch (+)-Biotin gehemmt wird. Die Dauer des antagonistischen Effekts beträgt 240–312 Stunden. Erst nach dieser Zeitspanne wird in Substrat C eine Steigerung der Wuchsstoffwirkung des meso-Inosits durch (+)-Biotin bewirkt. In diesen Versuchen schwankte die Durchlässigkeit der Biotin-Inosit-Kulturen unmittelbar nach der Impfung zwischen 93,6 und 99,2%. In Nährlösung B^1 lässt sich der Antagonismus nur bei Verwendung kleiner Impfmengen ($D \cong 99\%$) feststellen, dagegen nicht bei mittlerer bis starker Impfung ($D \cong 97\%$). Im letzten Falle setzt die steigernde Wirkung des (+)-Biotins sofort nach der Impfung ein.

Ob diese Unterschiede auf die Art der Stickstoffquelle (Amino- bzw. Ammonstickstoff) zurückzuführen sind, lässt sich beim jetzigen Stand der Untersuchungen nicht entscheiden. Immerhin zeigen unsere Ergebnisse eindeutig, dass der Wuchsstoffbedarf der Hefen nur unter Berücksichtigung der jeweiligen Zusammensetzung des Substrats definiert werden darf. In diesem Zusammenhang sei daran erinnert, dass bereits WILLIAMS und Mitarb. die Bedeutung des Asparagins und der Asparaginsäure für die fördernde Wirkung der (+)-Pantothensäure sowie des β-Alanins auf die Vermehrung gewisser Hefestämme nachweisen konnten (siehe WILLIAMS und ROHRMAN (5), SCHOPFER (1) S. 127).

Die Untersuchungen über die Physiologie der Weinhefen werden von der Abteilung für Landwirtschaft des Eidg. Volkswirtschaftsdepartements aus dem Weinbaufonds unterstützt. Den zuständigen Behörden sprechen wir unseren herzlichsten Dank aus.

Die „Dézaley"-Hefe wurde dem hiesigen Institut von den „Stations fédérales d'essais viticoles, arboricoles et de chimie agricole", Lausanne, zur Verfügung gestellt. Herrn Direktor Dr R. GALLAY sowie Herrn Dr C. FLEURY möchten wir an dieser Stelle für ihre Liebenswürdigkeit herzlich danken.

Fräulein L. BENDEL danken wir bestens für sorgfältige Assistenz.

Summary.

In preceding articles (3, 4) the authors reported the results obtained in investigating the growth of the selected Swiss culture yeasts Fendant, Herrliberg and Salenegg, employed in preparation of wines at low temperatures. It was demonstrated conclusively

that these tame yeasts are autotrophic for growth factors, normal growth being obtained without addition of vitamins in nutrient solutions containing glucose, mineral salts, and ammonium sulfate (substrate B^1) or casein hydrolysate (substrate C) as a source of nitrogen. Thus it is incorrect to generalize the theory of auxo-heterotrophy of tame yeasts.

Addition of (+)-biotin does not induce any increase in rate of growth, nor in maximum yield of cells in cultures of the races Herrliberg and Salenegg, whereas the same vitamin in amounts greater than 0.0025–0.025 γ per 1000 ml of substrate has a very small beneficial action upon growth of the Fendant yeast. On addition of meso-inositol in quantities greater than 0.25–2.5 mg per 1000 ml a marked increase in growth of the former races is produced, whereas the stimulating effect upon the Fendant yeast is only a very slight one. None of the auxo-autotrophic tame yeasts mentioned is affected by pyridoxine, thiamin or (+)-pantothenic acid, added singly or in various combinations.

The present paper describes the results of studies on the growth requirements exhibited by the selected Swiss wine yeast Dézaley in the above synthetic nutrient solutions. The yeast was grown at 20° C. in culture tubes containing 8 ml of substrate. The tubes were shaken thoroughly once a day. The absorption of light in the Beckman photoelectric quartz spectrophotometer at the wave length 660 mμ was used as a measure of growth. The photometer readings in percentage transmission are given in the tables showing yeast growth as a function of time (3).

The growth experiments showed conclusively that the Dézaley yeast is an auxo-heterotrophic strain requiring the simultaneous addition of three vitamins for normal growth, namely (+)-biotin, meso-inositol, and (+)-pantothenic acid. The growth responses found for the Dézaley cells in the above media C (casein hydrolysate) and B^1 (ammonium sulfate) with various supplements of vitamins may be summarized as follows (washed inoculum from substrate A; cf. (3)).

Medium C (alone): No growth within 240–500 hours. Slow growth after incubation for longer periods. The length of the time thus required for start of growth in the vitamin-deficient substrate C depends upon the quantity of inoculum used.

Medium C + (+)-biotin: No stimulation of growth within 200–600 hours. After incubation for 600–700 hours a beneficial effect.

Medium C + pyridoxine or thiamin: No growth-promoting action.

Medium C + (+)-biotin + pyridoxine or (+)-biotin + thiamin: No beneficial action upon growth.

Medium C + (+)-pantothenic acid: Stimulation of growth after 75–150 hours. The growth curve, however, is flat. The yield of cells observed after very long times of incubation (300–900 hours; 2.5 mg of calcium pantothenate per 1000 ml of substrate) is small as compared with that attained after short incubation periods in presence of the optimum combination of vitamins.

Medium C + meso-inositol: Stimulation of growth without lag phase. The maximum yield of cells obtained after incubation for 200–900 hours in presence of meso-inositol alone is small as compared with that produced after only 70–120 hours in cultures containing simultaneously (+)-biotin, meso-inositol and (+)-pantothenic acid.

Medium C + meso-inositol + (+)-biotin: Within a certain period ($\leqq$ 250–300 hours) the growth-promoting action of meso-inositol is inhibited by (+)-biotin. The yeast cells start growth without lag but stop growing within 24 hours at a level depending on the size of inoculum used. In presence of 25 mg of meso-inositol per 1000 ml of substrate the minimum concentration of (+)-biotin producing a marked antagonistic effect amounts to 0.025 γ per 1000 ml. Upon prolonged incubation ($\geqq$ 250–300 hours) the cultures containing both vitamins show an increase in rate of growth and maximum yield of cells as compared with the cultures supplied with meso-inositol alone.

Medium C + (+)-pantothenic acid + (+)-biotin: Stimulation in rate of growth and increase in maximum yield of cells as compared with the cultures supplied with (+)-pantothenic acid alone.

Medium C + meso-inositol + (+)-pantothenic acid: The cultures supplied with both vitamins manifest a considerable increase in rate of growth as compared with those containing only one of the growth factors.

Medium C + (+)-biotin + meso-inositol + (+)-pantothenic acid: Excellent growth is obtained on simultaneous addition of 25 γ of (+)-biotin, 25 mg of meso-inositol, and 2.5 mg of calcium pantothenate per 1000 ml of substrate. The rate of growth and maximum yield of cells observed in presence of this combination of growth factors are equal to those obtained for the auxo-autotrophic tame yeasts Fendant, Herrliberg and Salenegg in vitamin deficient media or in presence of meso-inositol alone (3, 4). Thus the Dézaley yeast is to be classed among the auxo-heterotrophic organisms requiring a comparatively complex constellation of vitamins for normal growth (cf. SCHOPFER (1), p. 205).

Medium B^1 (alone): Slow growth after 48–120 hours. Thus substrate B^1 is more favourable than substrate C for development of the Dézaley yeast. The significance of this difference in growth to the sources of nitrogen concerned (ammonium sulfate and casein hydrolysate, respectively) is not clear from the results described in the present paper. Further it is a matter of question whether the reduction of the lag in the vitamin-deficient substrate B^1 is due to a stimulation of a slow synthesis of vitamins taking place in all yeast cells, or involves a selective process whereby a limited number of cells with auxo-autotrophic tendencies, present in the overwhelming population of auxo-heterotrophic cells, start multiplying at an accelerated rate. In addition to these noninherited adaptation and selection mechanisms genic mutations may be involved in the eventual changes of the population equilibrium occurring in the media B^1 and C.

Medium B^1 + (+)-biotin: Strong stimulation of rate of growth after 215–260 hours and a very high maximum yield of cells. Thus the lag or induction period is reduced as compared with that observed for the corresponding cultures in medium C (in both cases 25 γ (+)-biotin per 1000 ml of substrate).

Medium B^1 + (+)-pantothenic acid: A beneficial action on growth after incubation for 265 hours or longer periods. Thus the lag is much longer than that obtained for substrate C on addition of an equal amount of (+)-pantothenic acid (2.5 mg calcium pantothenate per 1000 ml of substrate). The yield of cells attained after 300–900 hours is very low as compared with that observed after only 170 hours in cultures containing the optimum combination of vitamins.

Medium B^1 + meso-inositol + (+)-biotin: With heavy inoculum no antagonistic effect but, without induction period, an increase in rate of growth as compared with that observed for the cultures containing only meso-inositol. When a light inoculum is used, the cultures behave like those in medium C. Thus a strong growth inhibition is obtained with short periods of incubation, whereas on prolonged incubation a marked beneficial effect on rate of growth and maximum yield of cells is observed.

On addition of meso-inositol alone, meso-inositol + (+)-pantothenic acid, (+)-biotin + (+)-pantothenic acid or (+)-biotin + meso-inositol + (+)-pantothenic acid, the cultures in medium B[1] grow in principle like those in medium C.

From the above we may conclude that the sporogenous tame yeasts used for production of wines form a highly heterogenous group as regards the need for an exogenous supply of vitamins. The Fendant, Herrliberg and Salenegg races show excellent growth in vitamin-free synthetic media, whereas the Dézaley yeast is dependent upon an exogenous source of at least three vitamins for normal growth. Current investigations in this laboratory indicate the possibility of grouping other wine yeasts on basis of an increasing degree of auxo-heterotrophy.

Literatur.

1. W. H. Schopfer, Plants and Vitamins. Waltham, Mass. 1949. – 2. N. M. Stelling-Dekker, Die sporogenen Hefen, Amsterdam 1931. – 3. T. Wikén und O. Richard, Antonie van Leeuwenhoek **17**, 209, 1951. – 4. T. Wikén und O. Richard, Antonie van Leeuwenhoek **18**, 31, 1952. – 5. R. J. Williams and E. Rohrman, J. Amer. Chem. Soc. **58**, 695, 1936.

(Medical Clinic of the University at Utrecht).

COMPARISON OF TWO METHODS FOR THE DETERMINATION OF THE RESISTANCE OF *MYCOBACTERIUM TUBERCULOSIS* TO p-AMINOSALICYLIC ACID AND STREPTOMYCIN

by

H. J. OSKAM

(Received April 26, 1952).

INTRODUCTION.

Resistance determinations on bacteria in the laboratory for clinical bacteriology have to be done in the shortest time possible. Especially in surgical treatment of tuberculosis previous measuring of the sensitivity of the tubercle bacteria to p-aminosalicylic acid (P.A.S.) and streptomycin is indispensable. The clinician is, however, less interested in the absolute values of these resistances than in their meaning in relation to concentrations attainable *in vivo*; he will, therefore, especially in tuberculosis, readily accept a technique which yields a profit of time even when it is at the cost of a small part of exactness.

Determinations as mentioned are not so difficult, if only a pure culture is available, which makes a loss of at least some weeks inevitable. "Direct" determination is frequently impaired by contaminating organisms. If a routine medium, such as Loewenstein's, should prove usable for such determinations a considerable gain of time could be obtained. The investigations described here tend to make a comparison of the Loewenstein-technique (9) with the technique using Herrold's egg-yolk medium (6) as described by KARLSON (7); this comparison has been made from indirect determinations.

Both media are solid: determinations in liquid media are still more susceptible of contaminations than on solid ones and have nevertheless to be carried out as surface cultures, unless surface active substances like "Tween-80" are used. Such substances may, however, considerably influence the sensitivity of the bacteria [Williston and Youmans (11)]. A much more important fact is the intrinsic difference of the results, obtained by a liquid and by a solid medium. In the first case the maximum resistance, occurring in the bacterial population under study, is measured, which only belongs to a varying, but often very small, number of bacteria. On solid media these bacteria will only produce single colonies, which do not influence the reading of the whole importantly. According to Welsch (10) these resistant individuals can be met with in every strain and, consequently, the resistance measured in a liquid culture will be the higher the larger the inoculum is. Evidently the size of the inoculum is on a solid medium of less importance as long as a certain minimal quantity of bacteria is present, after Colwell c.s. (4) about 10^{-6} mg. This is important, because in pathological specimina the inoculum cannot be standardized. In direct resistance determinations it is only necessary that a certain number of colonies is present in the control tubes, 25 at least (9).

Every solid medium can be used if its preparation does not include an important destruction of the antibiotic which is added. Preferably the same medium should be chosen as is used for the routine cultivation of *M. tuberculosis* from pathological materials. It is only for this reason that we prefer Loewenstein's medium to those described by others, e.g. Beeuwkes (1) and Coletsos (3).

On the rich medium of Herrold the slightest contamination often means a complete failure; it can, therefore, practically only be used for indirect determinations. In Loewenstein's medium on the other side, the heating might do serious harm to the antibiotics; moreover the malachite green, being a bacteriostatic substance, might influence the results. The following data are obtained first by investigation of the results on Herrold's medium and secondly by comparison of the resistances of strains tested in both media.

TECHNIQUE.

In the medium of Herrold-Karlson the antibiotic is added together with the egg-yolk and glycerol to the previously sterilized nutrient agar (Bacto) at about 45°C. To the Loewenstein-medium it is added before the heating for coagulation, which lasts 80 minutes at about 82°C. The use of fresh eggs and a good aseptic preparation warrant excellent results by heating only one time. Heating after the Copenhagen scheme: 5 minutes at 90°C. followed by 20 minutes at 85°C. was not possible with the available equipment.

The media are prepared in series of the following concentrations: 1, 10, 50 and 100 micrograms/ml of sodium-p-aminosalicylate, calculated as pure P.A.S., and 1, 5, 10, 50 and 100 micrograms/ml of dihydrostreptomycin.

The inoculum, its exact size being of minor importance, is prepared by shaking the whole surface growth of a Loewenstein culture, taken up in 2 ml of physiological saline, in a flask with glass beads for 20 minutes by means of a shaking apparatus. The tubes are inoculated with 3 drops of the suspension: after having let them flow over the surface of the medium together with the condensation fluid, the tubes, which are closed with a rubber stopper, are incubated in upright position at 37°C.

Reading is done after 21 days of incubation, preferably with the naked eye. Stained preparations are only made in dubious cases; to judge them a considerable amount of experience is necessary. The finding of acid-fast rods is, by itself, not a proof of the existence of "subvisible" growth; conclusions can only be based on morphology. Only if cords or strings can be clearly distinguished it is allowed to speak of "colonies naissantes" [COLETSOS (3)]. Now and then the higher concentrations show floccules, which, microscopically, consist of swollen and granulated elements. Sometimes, however, these floccules are real small colonies, examples of sporadic growth (in the tables indicated as "sp"). It could not be confirmed that this sporadic growth is always present [WELSCH (10)] and we do not know whether occurring or not occurring of this phenomenon means a greater or smaller tendency of a particular strain to develop resistance; it can only be said that it may regularly return in a series of cultures from the same patient.

To indicate the obtained results the following notations are used: no growth except on controls: N.R. (= non resistant); growth on 1, but not on 5 micrograms/ml: 1/5 and so on. Often more than one resistance level can be distinguished: in such cases only the highest level is mentioned.

RESULTS.

Tables I and II show the results of a number of resistance determinations in duplo on Herrold's medium: two specimina were taken from the same patient with an interval varying from 1 day to 3 weeks.

TABLE 1.

Double P.A.S.-resistance determinations on Herrold's medium in strains cultivated from the same patient with short time intervals. For explanation of abbreviations see text.

1st det.	2nd det.	conform	sp. diff.	more diff.
N.R.	N.R.	6		
N.R.	N.R., sp		2	
N.R.	1/10			1
N.R.	10/50			3
N.R., sp	1/10			1
N.R., sp	1/10, sp			1
N.R., sp	> 100			1
1/10	1/10	1		
1/10	10/50			3
1/10, sp	1/10, sp	1		
1/10, sp	> 100			1
10/50	10/50	2		
10/50, sp	> 100			2
50/100	50/100	1		
50/100	> 100			2
> 100	> 100	11		
39	39	22	2	15

TABLE 2.

The same as table I, but dealing with streptomycin-resistances.

1st det.	2nd det.	conform	sp. diff.	more diff.
N.R.	N.R.	1		
1/5	1/5	10		
1/5	1/5, sp		1	
1/5	5/10			2
1/5	10/50			1
1/5, sp	1/5		1	
5/10	1/5			3
5/10	5/10	3		
5/10	10/50			1
10/50	1/5			1
10/50	5/10			1
10/50	10/50	2		
> 100	> 100	1		
28	28	17	2	9

Table I (P.A.S.-resistances) shows in 39 of such pairs 22 (56 %) or, neglecting the difference "sp", 24 (62 %) conform readings, differences of one interval in 8 (20 %), of more than one interval in 7 (18 %) cases.

Table II (streptomycin-resistances) shows in 28 double determinations 17 (61 %), respectively 19 (68 %), conform readings and differences of one interval or more in 7 (25 %) and 2 (7 %) cases respectively.

Undoubtedly these differences cannot be regarded as mere failures of the technique. Local differences of the composition of the bacterial population must play an important rôle. The phenomenon has — at least in relation to streptomycin resistance — been described by others, e.g. Canetti and Saenz (2), Courmont, Galy and Déries (5) and Linz (8). It is, therefore, commendable to take much material and to carry out 2 or 3 determinations with short intervals of time.

In tables III and IV pairs of determinations are given, done from one culture, following the two techniques described.

TABLE 3.

P.A.S.-resistances simultaneously determined in the media of Herrold (H) and of Loewenstein (L) in the same strain.

H	L	conform	sp. diff.		more diff.	
			H higher	L higher	H higher	L higher
N.R.	N.R.	16				
N.R.	N.R., sp			6		
N.R., sp	N.R., sp	8				
1/10	1/10	1				
1/10	1/10, sp			1		
1/10, sp	1/10, sp	3				
10/50	10/50	1				
> 100	10/50				1	
> 100	> 100	9				
46	46	38	0	7	1	0

Table III (P.A.S.-resistances) shows conformity in 38 (83 %), respectively 45 (98 %) out of 46 cases, whereas an appreciable difference is only seen in 1 case. It has to be admitted, that all of the 7 cases (15 %) showing "sp" difference have the higher readings on

TABLE 4.

The same as table III, but dealing with streptomycin-resistances.

H	L	conform	sp. diff.		more diff.	
			H higher	L higher	H higher	L higher
N.R.	N.R.	1				
1/5	1/5	21				
1/5	1/5, sp			8		
1/5	5/10					3
1/5	5/10, sp					1
1/5, sp	1/5		1			
1/5, sp	1/5, sp	1				
1/5, sp	5/10, sp					1
1/5, sp	10/50					1
5/10	5/10, sp			2		
5/10, sp	5/10, sp	1				
5/10, sp	> 100					1
10/50	5/10				1	
10/50, sp	1/5, sp				1	
10/50, sp	5/10, sp				1	
45	45	24	1	10	3	7

the side of the Loewenstein, but the much better readableness on its green background is partly responsible for it. Obviously the two techniques can replace each other completely.

Table IV (streptomycin-resistances) gives somewhat less favourable results: out of 45 pairs of determinations only 24 (53 %), respectively 35 (78 %), show complete conformity. In the remaining 10 cases the higher reading is 3 times on the side of Herrold and 7 times on the side of Loewenstein. These numbers are too small to allow statistical conclusions, but it is clear, that the differences are more a consequence of a less exact readableness of streptomycin-resistances than of a decrease of activity of the streptomycin in the medium.

DISCUSSION.

Summarizing the results of the 4 series of experiments the following conclusions can be made:

1°. In double determinations, following the same technique, but carried out on different specimina from one patient, differences can be met with, which are greater than the differences found by testing one culture following the two techniques.

2°. As for the P.A.S. there is no decrease of activity in the Loewenstein-medium; as for the streptomycin it may be present, but it is too small to produce significant differences in the results. As can be seen from tables II and IV, most strains show a streptomycin-resistance of 1/5. Probably part of these strains don't differ from N.R. strains importantly. It will be useful to extend the series of streptomycin concentrations with a tube containing 2 micrograms/ml.

3°. As Loewenstein's medium is more suitable for isolation of *M. tuberculosis* from pathological specimina, the results described above justify the choice of this medium for resistance determinations, direct and indirect.

Summary.

A comparison is made of resistance determinations of *M. tuberculosis* on Loewenstein's and on Herrold's medium. No appreciable differences could be found. Loewenstein's medium is preferred because of its greater suitability for direct isolation of the bacteria and, consequently, for direct resistance determinations.

Literature.

1. H. Beeuwkes, Antonie van Leeuwenhoek **16,** 359, 1950. – 2. G. Canetti et A. Saenz, Ann. Inst. Past. **80,** 238, 1951. – 3. P. J. Coletsos, H. Boisvert et E. Oriot, Ann. Inst. Past. **79,** 288, 1950. – 4. C. A. Collwel, G. Pitner and M. Moravec, Am. Rev. Tbc. **63,** 679, 1951. – 5. P. Courmont, P. Galy et G. Déries, C.R. Soc. Biol. **144,** 4, 1951. – 6. R. D. Herrold, J. Inf. Dis. **48,** 236, 1931. – 7. A. G. Karlson, Proc. Staff. Meet. Mayo Clin. **23,** 401, 1948. – 8. R. Linz, C. R. Soc. Biol. **145,** 935, 1951. – 9. Personal informations of the Tuberculosis Department of the Statens Serum Institut, Copenhagen, Denmark. – 10. M. Welsch, C. R. Soc. Biol. **143,** 1401, 1949. – 11. E. H. Williston and G. P. Youmans, Am. Rv. Tbc. **59,** 336, 1949.

(From the Division of Food Technology, College of Agriculture, University of California, Berkeley, California).

CHARACTERISTICS OF TREHALASE IN *CANDIDA TROPICALIS*

by

T. M. LUKES and **H. J. PHAFF**

(Received March 28, 1952).

Trehalose is one of the important reserve carbohydrates in yeast. The literature on its occurrence in yeast was recently reviewed by MYRBÄCK (9) and BOUTHILET *et al.* (2). The utilization of stored trehalose by growing cells and the fermentation of added trehalose, appears to involve, at least in part, the enzyme trehalase, since its activity has been demonstrated in various strains of bakers yeast.

The enzyme was discovered in yeast by FISCHER (4). Other early studies dealing with yeast trehalase were reported by KALANTHAR(6) and BAU (1) who found the enzyme to be very unstable and not extractible from the cells. MYRBÄCK and coworkers in a series of more recent papers (7, 8) studied some of the characteristics of yeast trehalase. They showed that the bulk of the trehalase in bakers yeast is bound to the insoluble cell material and that only a small part goes into solution during autolysis. However, no systematic studies on the solubilization of the enzyme were reported. Activity determinations were based on measurements of increase in reducing value of trehalose solutions. Unfortunately only initial and final values were determined, which, at best, have only approximate quantitative value. Reports dealing with optimum pH and other properties have therefore only limited significance.

The ability of various yeasts to ferment added trehalose by fresh cells was reported in a previous paper (2). It was found that 16 species representing 7 genera were able to ferment this sugar. One culture, identified as a strain of *Candida tropicalis*, fermented trehalose more vigorously than the other yeasts, especially when the organism was grown on a slant containing this sugar as the sole source of carbon. Since there is a lack of information in the literature on the properties of trehalase extracted from yeast, a method was developed to prepare trehalase in soluble form from *Candida tropicalis*. After developing a reliable method of activity determination, the effect of pH and temperature on the activity and stability of the enzyme were studied.

METHODS AND MATERIALS.

Determination of trehalase activity. The increase in reducing value of the non-reducing disaccharide was followed as a function of time using the ceric sulfate method of HASSID (5). Equal volumes of the enzyme preparation (adjusted to pH 4.5 with McIlvaine's buffer) and a 1% aqueous solution of C.P. trehalose dihydrate were mixed and the reaction was followed at 30° C. Blank values, taken at zero time, were subtracted from the reducing values found. The amount of glucose liberated was plotted and was found to be a linear function of time during the first 60—70% of the hydrolysis. The rate of hydrolysis was found to be proportional to the enzyme concentration. This is evidenced by the fact that three enzyme solutions (a) undiluted, (b) diluted 1 : 1 and (c) diluted 1 : 4 gave activities of 100%, 51.0% and 20.4% respectively. When suspensions were used containing cell debris, the reaction vessels were placed on a shaker. One unit of trehalase activity was defined as the amount of enzyme which at pH 4.5 and 30° C. produces 1 mg of glucose per minute in a 0.5% solution of trehalose dihydrate. The following cultures were included in the study:

Candida tropicalis (No. 413)
Nematospora coryli (No. 31)
Debaryomyces globosus (No. 69)
Saccharomyces cerevisiae (No. 299)
Saccharomyces cerevisiae (No. 420)

The first four cultures can ferment added trehalose (2). Culture 420 slowly utilized trehalose, but does not ferment it. The *Saccharomyces* species were included, since the trehalase dealt with in earlier publications was nearly always present in strains of bakers or brewers yeast.

The yeasts were first grown on glucose yeast autolysate slants (2) for 2 days after which a sterile water suspension of the growth was inoculated onto yeast autolysate agar, containing 5% of the desired carbohydrate and allowed to grow for 2 to 3 days at 30° C. After growth, the yeast was washed and centrifuged twice with distilled water. The content of solids was determined in an aliquot by drying in a vacuum oven at 70°C. and a measured volume of the remainder centrifuged again and made up to a known volume (approximately three times that of the wet yeast) with the desired buffer solution. Chloroform was used as an autolysing agent. After autolysis (specified times at 30° C.) the cell debris was centrifuged and sediment and supernatant made up to a known volume for determination of enzyme activity.

RESULTS.

The five yeasts listed above were grown for 2 days at 30° C. on 5% glucose yeast autolysate agar. After harvesting the cells as described before, the sediment was suspended in 1% KH_2PO_4 (pH 4.6) and autolysed for 5 days at 30° C. with the aid of chloroform. The activities in the supernatant and cell debris expressed per unit dry weight of the original cells are given in Table 1.

TABLE 1.

Distribution of trehalase between cell debris and autolysate of various yeasts grown on glucose yeast autolysate agar.

Species of yeast	Fraction of autolysate	Units of activity per g dry yeast × 300
Sacch. cerevisiae 420	cell free	7.9
	cell debris	19.5
Sacch. cerevisiae 299	cell free	5.0
	cell debris	48.5
Candida tropicalis	cell free	12.4
	cell debris	79.3
Nematospora coryli	cell free	13.9
	cell debris	27.8
Debaryomyces globosus	cell free	3.29
	cell debris	18.4

The figures show that the highest trehalase activity was present in *Candida tropicalis*. Our results, therefore, fit in well with the findings of Bouthilet *et al.* (2), that *C. tropicalis* was the strongest trehalose fermenting yeast among a considerable number of species tested. It also suggests strongly that the fermentation of trehalose takes place after a preliminary hydrolysis to glucose by the enzyme trehalase.

The observations of Myrbäck and Örtenblad (7), that trehalase in bakers yeast is attached for the most part to the cell debris, was confirmed for *S. cerevisiae*, and was also found true for the other three yeasts. The percentage of activity retained on the cell debris varied somewhat, ranging from 90.8% for *S. cerevisiae* 299 to 66.7% for *N. coryli*.

Since Bouthilet *et al.* (2) showed that trehalase was an adaptive enzyme in *C. tropicalis*, the above experiment was repeated by growing the 5 yeasts on a similar medium, except that the carbon source was changed to 4% glucose + 1% trehalose. It was found that the total trehalase content of the two *S. cerevisiae* strains was not stimulated, that of *N. coryli* about 10%, of *D. globosus* about 25% and *C. tropicalis* 30% by incorporating 1% trehalose in the medium. *C. tropicalis* was also grown on yeast autolysate agar containing 5% trehalose as a carbon source. Under these conditions the trehalase activity increased 8.5 times as compared to that when the yeast was grown on glucose alone.

Autolysis under controlled conditions.

In the experiments reported in Table 1, the pH was not kept constant during autolysis. The latter causes the liberation of basic substances and due to the relatively poor buffering capacity of the KH_2PO_4 present, the pH rose from 4.6 to about 5.8. In another experiment in which the pH was maintained at 4.5 by the addition of acid, it was found that only the cell debris contained activity. Even when the autolysis was continued for 12 days no activity was found in the solution. This observation induced us to study in more detail the effect of pH on the liberation of trehalase from the cells. McIlvaine's phosphate-citrate buffer was used to establish the pH values 4.5, 5.5 and 6.3. Higher values are not reported, because trehalase was found to decompose rapidly during autolysis at pH 7.0. Especially at the lower pH levels it was necessary to add some dilute HCl to keep the pH constant. The 1% KH_2PO_4

solution, used in the initial experiments, was also included for comparison. The results are given in Table 2.

TABLE 2.

Distribution of trehalase between cell debris and autolysate of *Candida tropicalis* during autolysis at several pH values.

Conditions of autolysis	Activity 2nd day per unit volume		Activity 5th day per unit volume	
	cell debris	cell free	cell debris	cell free
KH_2PO_4(pH4.7→5.8)	154	9.5	135	21.5
pH 4.5	137	0.0	114	0.0
pH 5.5	125	14.5	104	25.7
pH 6.3	41	47.5	27	71.5

The same amount of yeast was used for each test, so that the activities are comparable. The figures show clearly that with increasing pH and time more trehalase went into solution. At pH 6.3 some loss in activity appeared during the first two days. This may have been caused by a slight rise in pH before the latter was readjusted to 6.3.

A more extensive test was then carried out in the hope to get more information on the mechanism of trehalase liberation from the cell debris. An example of such an experiment is outlined in figure 1.

Table 3 contains the activities of the various fractions. The activity figures of cells and supernatant during autolysis at pH 6.3 show, that when time progressed an appreciable activity appeared rather rapidly in the supernatant solution and that of the cell debris decreased. After the first three days the rate of solubilization of trehalase slowed down appreciably. The possibility was considered that there might be a factor in the autolysate (possibly an enzyme) responsible for the solubilization of trehalase during autolysis at pH 6.3 and that this factor became inactivated or was destroyed gradually during autolysis, thus slowing down the solubilization of trehalase. Evidence, that it was not merely the pH of 6.3 which was responsible for solubilization of trehalase, was furnished by the fact, that during storage in pH 6.3 buffer of the D fraction (containing full activity on the cell debris), no trehalase went into

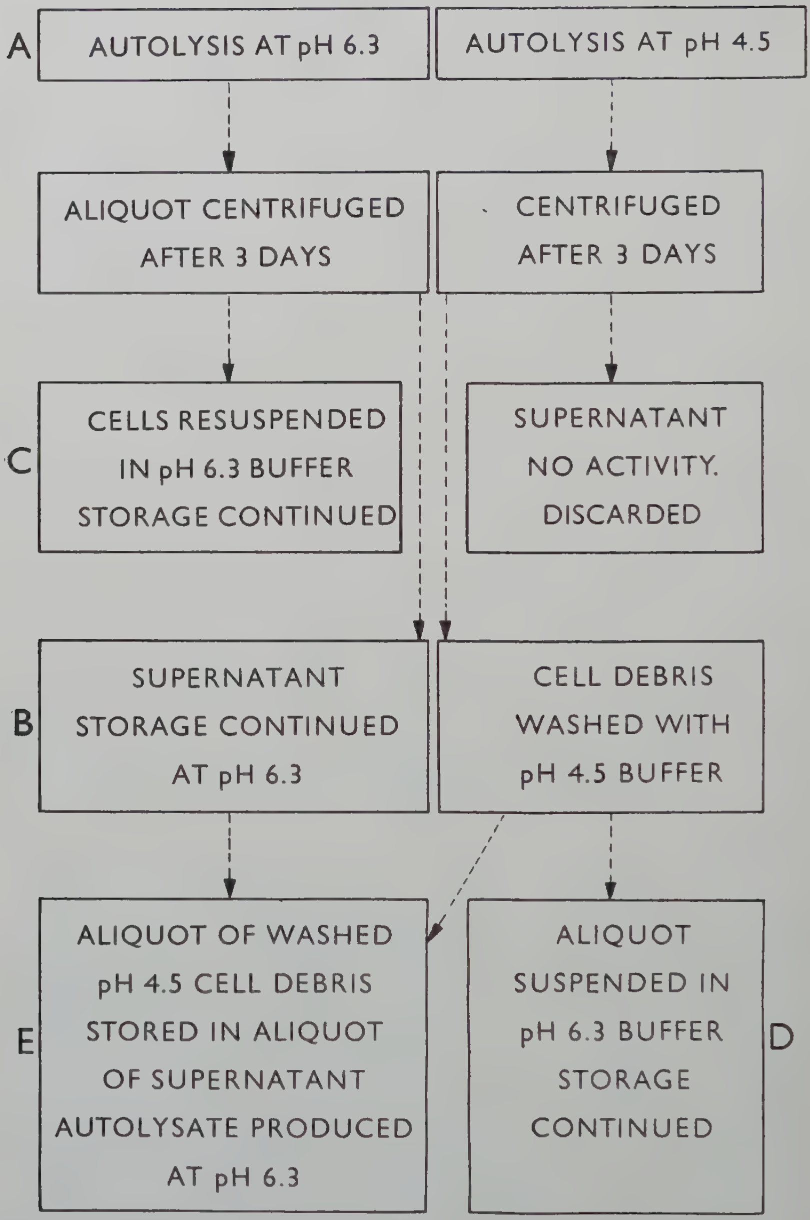

A
AUTOLYSIS AT pH 6.3
AUTOLYSIS AT pH 4.5
ALIQUOT CENTRIFUGED AFTER 3 DAYS
CENTRIFUGED AFTER 3 DAYS
C
CELLS RESUSPENDED IN pH 6.3 BUFFER STORAGE CONTINUED
SUPERNATANT NO ACTIVITY. DISCARDED
B
SUPERNATANT STORAGE CONTINUED AT pH 6.3
CELL DEBRIS WASHED WITH pH 4.5 BUFFER
E
ALIQUOT OF WASHED pH 4.5 CELL DEBRIS STORED IN ALIQUOT OF SUPERNATANT AUTOLYSATE PRODUCED AT pH 6.3
ALIQUOT SUSPENDED IN pH 6.3 BUFFER STORAGE CONTINUED
D

Fig. 1. Autolysis of C. tropicalis cells at pH 6.3 and 4.5. Cell debris and supernatant liquids treated in various ways. For activities of trehalase in the different fractions see Table 3.

solution. Additional evidence, that the solubilization factor became less active during the progress of autolysis, is the observation that during storage of the E fraction (pH 4.5 cell debris carrying full activity, mixed with an aliquot of the 3 day old supernatant of a pH 6.3 autolysis) only a relatively modest amount of enzyme went into solution.

TABLE 3.

Relative activities of cell debris and supernatant of the fractions described in figure 1 during storage at 30° C. All activities are comparable and based on the same unit volume.

Fraction	Activities after various times					
	4 hrs.	1 day	3 days	4 days	5 days	11 days
A cell debris	247	215	147		133	78
A supernatant	0	78	210		191	129
A total activity	247	293	357		324	207
B supernatant			244		234	246
C cell debris			120		122	133
D cell debris				272		276
D supernatant				0		0
E cell debris				310		284
E supernatant				180		242
E total activity				490		526

The activity figures of fractions A and E (Table 3) during autolysis show a greater increase in soluble enzyme than the corresponding loss in insoluble enzyme. This would indicate that maximum activity is not demonstrated by the insoluble trehalase (possibly because of limited contact between enzyme and substrate), even though an initial linear relationship between per cent hydrolysis and time was found.

The data of fraction A (Table 3) also show that ultimately, when autolysis was extended to 11 days and cell debris and autolysate were allowed to remain in contact with each other, a fairly appreciable loss in enzyme activity took place. However, when cell debris and supernatant were separated from each other (fractions

B and C) and stored individually at the same pH, no loss in activity occurred during the next 8 days of incubation. This observation might be explained by postulating a separate insoluble proteolytic enzyme present in the cell debris able to destroy soluble trehalase, but not insoluble trehalase present on the same cell debris. Lack of contact with the substrate may account for the latter. Examples of this are known in the literature, for example the existence of trehalose and trehalase together in living yeast cells. Thus the stability of fraction B can be explained on the basis that the cell debris had been removed and fraction C was stable because both enzymes are assumed to be insoluble on the debris, preventing proper contact with each other. This postulated proteolytic enzyme on the debris might also be responsible for the gradual destruction of the solubilization factor. Thus it appears that the crude trehalase preparations are reasonably stable at pH 6.3 and their instability during autolysis at the same pH appears to be due to secondary causes.

The alcohol precipitation of trehalase.

The previous experiments have shown that soluble trehalase can be produced by autolysis at pH 6.3 for 3 to 4 days at 30° C. It was desirable to purify the enzyme partly and to concentrate it, since in the crude autolysate preparations a rather large amount of reducing material was present, which complicated the trehalase assay. This difficulty was also experienced by Myrbäck and Örtenblad (7). The suggestion of Cohn *et al.* (3) was followed, that protein fractionation be carried out with alcohol at as low a temperature as possible and under controlled pH conditions. The pH was adjusted to various levels between 4.0 and 5.0, since a rough test had indicated that the optimum pH of trehalase was in this range and therefore in all likelyhood also the iso-electric point of the enzyme. The enzyme solutions were cooled to 0° C. and enough ethanol, precooled to —18° C., was added slowly under rapid stirring to reach the desired concentration. After standing for 24 hours at —18° C., the precipitates were centrifuged at 0° C. and redissolved in pH 4.5 buffer and made to the same volume as before. The results are presented in Table 4.

The figures show that 65% ethanol gave the highest recoveries, but there was also a large amount of inert protein precipitated at

TABLE 4.

Per cent recovery of trehalase at various alcohol concentrations and different pH values during precipitation.

Alcohol conc. V/V after mixing	pH 4.0	pH 4.3	pH 4.5	pH 4.7	pH 5.0
35	17.0	—	4.1	—	12.3
40	51.0	41.0	—	38.0	44.0
65	67.0	65.0	—	77.0	59.0

this concentration. With the lower concentrations there was little material precipitated, but the activity per unit of dry matter was high. As a result precipitation at a concentration of 40% alcohol and pH 4.0 was used to purify trehalase. After redissolving in McIlvaine's buffer at pH 4.5, the solution appeared faintly opalescent and had very little reducing power.

Effect of pH and temperature on the stability of trehalase.

Equal portions of an alcohol purifed trehalase solution were adjusted to various pH values in McIlvaine's buffer, all made to the same volume and then incubated at 30° C. for one week with toluene as a preservative. After storage the pH was adjusted to 4.5 in all cases and the residual activity tested. The results are given in Table 5.

TABLE 5.

Stability of purified trehalase at various pH values and 30° C. for 1 week.

pH of storage	3.65	4.50	5.3	6.3	7.4
per cent of original activity left	60.0	84.2	84.2	43.0	0.0

The purified enzyme appears to be most stable between pH 4.5 and 5.3 and the stability decreases more rapidly towards the alkaline side than the acid side. The purified enzyme seems to be much less stable at pH 6.3 than the crude soluble enzyme at the same pH (see Table 3). However purified enzymes are frequently much less stable than crude preparations as for example in the case of invertase (10). Another purified trehalase preparation was stored at pH 4.5 and 0° C. The solution was preserved with toluene and the activity determined periodically. It was found that no measurable loss in activity took place up to 51 days of storage. This finding made it possible to have on hand a supply of stable soluble trehalase, when stored at 0° C.

The optimum pH of trehalase.

As mentioned earlier the optimum pH had been roughly determined to be near 4.5. A more accurate test was now made with the use of soluble trehalase. The enzyme was first dissolved in a small amount of 0.05 M citrate adjusted to pH 4.5. Aliquots were then pipetted into the much stronger McIlvaine buffers (0.15 M) of various pH values. The trehalose was added and the activity determined without delay because of the limited trehalase stability in the extreme pH ranges. The actual pH values for each test were determined at the end of each test period. The results are given in Table 6.

TABLE 6.

Relative activities in per cent of maxium of yeast trehalase at 30° C. and various pH values.

pH	2.70	3.65	3.95	4.5	4.95	5.30	5.50	6.30	7.40
Activity %	41	87	97	100	100	100	97	60	12

The data show that yeast trehalase has a broad optimum pH, approximately between 4.0 and 5.5. Myrbäck and Örtenblad (7) reported for bakers yeast an optimum value of 5 to 6 with a rather sharp peak at 5.5. Their method consisted simply of determining the increase in reducing power (only an initial and final figure reported) at the expence of stored trehalose during autolysis of fresh bakers yeast at various pH values in the presence of ethylacetate and NaF (the latter to prevent fermentation). Partly because the pH changed rather appreciably during the tests, their results cannot be considered very accurate. However, in spite of these shortcomings it is interesting that the curve on the alkaline side of the optimum checks very well with our results. On the acid side however there are differences since we found a much broader optimum. The trehalase of the mushroom *Lactarius torminosus* appears to have an optimum pH of about 4.0 according to Will-

STAEDT and BORGGÅRD (11). Since these authors made no rate measurements, their results must be considered approximate. It does appear from their data that at pH 5.3 the mushroom trehalase is vastly less active than at pH 4.1, whereas the trehalase of *C. tropicalis* has full activity at pH 5.3.

The effect of temperature on the activity of yeast trehalase.

Equal volumes of a solution of trehalase at pH 4.5 in McIlvaine's buffer and 1% trehalose in distilled water were mixed in a test tube after being brought approximately to the temperature to be tested. The tube was placed in a constant temperature oil bath and after 5 minutes of temperature adjustment, samples were withdrawn to study the rate of hydrolysis. At all temperatures reported the rate was a linear function of time during at least the first hour. At 60.5° C., the enzyme was inactivated so rapidly, that no accurate rate measurement could be made. The figures are reported in Table 7.

TABLE 7.

Relative activities in per cent of maximum activity of yeast trehalase at various temperatures.

Temperature °C.	20.0	30.5	40.5	43.5	48.0	50.5
Activity percent	16.7	46.5	88.0	97.0	100	97.0

The results indicate that the maximum rate was found at about 48° C. Between 20 and 30° C. the temperature coefficient (Q 10) was found to be 2.7 and from 30.5—40.5 C., 1.9.

Discussion.

The studies presented above have shown that certain yeast species contain appreciably greater quantities of the enzyme trehalase than bakers yeast which has been the subject of nearly all studies dealing with this enzyme. Observations of other workers that trehalase is tightly bound to cell debris were confirmed, but it was found that it is possible to release most of the enzyme from the cells by carrying out autolysis at a controlled pH of 6.3. When the pH is too low (4.5) no enzyme goes into solution, if the pH during autolysis is too high (above 7.0) the enzyme is rapidly destroyed. We have found some evidence for the existence of an enzyme associated with the cell debris, which at suitable pH values slowly destroys trehalase in solution. On the other hand, trehalase in crude preparations free from cell debris is quite stable in the autolysate from our yeast. After partial purification the stability of trehalase under comparable storage conditions is reduced, although at refrigeration temperatures no loss in activity was noticed in 51 days at pH 4.5. *Candida tropicalis* strain No. 413, offers a suitable source for the enzyme trehalase, particularly when grown in the presence of the corresponding sugar, since the enzyme is adaptive in this strain.

The experiments have also shown that maximum activity of the trehalase present in suspensions of cell debris is not reached, presumably because in this case of heterogeneous catalysis contact between substrate and enzyme is limited because of the concentration of the enzyme in certain distinct loci. In one case a total activity of 144% was found after solubilization of the trehalase as compared to that at the start of autolysis (Fraction A, Table 3).

Summary.

The trehalase content of different yeasts varies widely. A strain of *Candida tropicalis* was found to be the best source of this enzyme among the yeasts tested. The trehalase activity in this yeast could be increased 8.5 times by growing it on trehalose rather than glucose. Thus trehalase is an adaptive enzyme in *C. tropicalis*.

It was found that the amount of trehalase which could be solubilized increased with increasing pH during autolysis of the cells, none being released from the cell debris at pH 4.5 and most at pH 6.3. Some evidence was obtained to show that the solubilization was caused by an enzyme. The stability of trehalase under various conditions was studied. A partial purification was achieved by precipitation with 40% ethanol at a temperature of —18° C. The maximum temperature of the enzyme was 48° C., and the optimum pH ranged from 4.1 to 5.3.

Bibliography.

1. A. Bau, W. f. Brauerei **16,** 305, 1899; **32,** 143, 1915; Biochem. Z. **73,** 340, 1916. – 2. R. J. Bouthilet, N. E. Neilson, E. M. Mrak and H. J.

Phaff, Jour. Gen. Microbiol. **3,** 282, 1949. – 3. E. J. Cohn, L. E. Strong, W. L. Hughes Jr., D. J. Mulford, J. N. Ashworth, M. Melin and H. L. Taylor, Jour. Am. Chem. Soc. **68,** 459, 1946. – 4. E. Fischer, Ber. **28,** 1432, 1895. – 5. W. Z. Hassid, Ind. Eng. Chem. Anal. Ed. **9,** 228, 1937. – 6. A. Kalanthar, Z. physiol. Chem. **26,** 88, 1898. – 7. K. Myrbäck and B. Örtenblad, Biochem. Z. **291,** 61, 1937. – 8. K. Myrbäck und B. Örtenblad, Biochem. Z. **288,** 329, 1936; **292,** 230, 1937; K. Myrbäck, B. Örtenblad und K. Ahlborg, Enzymologia **3,** 210, 1937; K. Myrbäck und E. Vasseur, Z. physiol. Chem. **277,** 171, 1943. – 9. K. Myrbäck, Ergebn. Enzymforsch. **10,** 168, 1949. – 10. C. Neuberg and I. S. Roberts, Invertase, A Monograph. Scientific Report Series No. 4. Sugar Research Foundation, Inc. New York, Sept. 1946, page 61 – 11. H. Willstaedt and M. Borggård, Arkiv. Kemi, Mineral. Geol., B **23.** 1, 1946.

(From the National Institute for Public Health, Utrecht, Holland).

ON THE PURIFICATION AND ADSORPTION OF STAPHYLOCOCCUS TOXOID

by

A. TASMAN, A. C. BRANDWIJK, A. MARSEILLE, J. D. VAN RAMSHORST and **J. VAN DER SLOT**

(Received June 13, 1952).

In 1933 TIMMERMAN (12) published a monograph on staphylococcus toxin and antitoxin, in which he discussed extensively the preparation of this toxin. In addition he described his experiments and those of other investigators, which demonstrated the formation of staphylococcal antitoxin in the bloodserum of animals, injected with toxin.

BURNET (2), PARISH and CLARK (7), TIMMERMAN (13), RAMON(9) and RAMON, RICHOU and DJOURICHITCH (10) described the formation of staphylococcal toxoid from the corresponding toxin by formalin treatment, similar to the formation of diphtheria and tetanus toxoid. In 1937, in a paper by TIMMERMAN (14), the application of staphylococcus toxoid as a remedy was discussed. His investigation showed that treatment of various kinds of furunculosis with staphylococcus toxoid was successful in many cases. Treatment of acne, sycosis barbae, osteomyelitis and panaritia patients met with less success.

Since then further experiments tended to a favourable impression of the curative power of this therapeutic substance. It strikes us however that some patients with furunculosis react better to an autovaccine („Stockvaccine" resp.), than to a treatment with staphylococcal toxoid and vice versa.

Although the administration of a proper toxoid preparation nearly always induces staphylococcal antitoxin formation, this does not imply that this antitoxin formation is the only essential element in the fight against staphylococcal infections. Apparently also other factors sometimes play an important part.

Application of this remedy, which on the whole gives satisfactory results, meets with two objections, to wit, the relatively great number of injections required (seven at the least) and the strong local or general reactions that often occur after the injections. These are sometimes of such vehemence that many a patient can not endure the entire treatment, making an untimely cessation of the cure necessary.

With the favourable experiences with purified and adsorbed diphtheria and tetanus prophylactics (Diphtheria-P.T. and Tetanus-P.T.) in mind, we have attempted to purify the staphylococcal toxoid and to adsorb this purified product on aluminium phosphate. As a result of the latter treatment a considerable increase in antigenic power might be expected.

So the object of our investigations was to prepare a purified and adsorbed staphylococcus toxoid which after fewer injections and with less unpleasant local and general reactions, would effect equal or better immunity than the crude preparation.

HOLT (5) was the first to purify staphylococcus toxoid by a total salting-out process with ammonium sulfate. He was able to purify and concentrate this toxoid, obtaining some purification and good yields (80—90%).

BURNET and FREEMAN (3) precipitated the toxoid with acetic acid, while BOIVIN and IZARD (1) used trichloroacetic acid. WITTLER and PILLEMER (17) purified the staphylococcus toxoid applying fractional precipitation in methanol-water mixtures at accurately controlled temperature, pH and ionic strength. These precipitations are carried out at temperatures below the freezing point.

In connection with our experience with the purification of diphtheria toxoid (VAN RAMSHORST (11)) we discarded the idea of isolating the staphylococcus toxoid by acid precipitation, because we found with the diptheria toxoid that, although acid precipitation often gives very good purification, the antigen is more or less damaged, which among other things is apparent from the increase in flocculation time of toxoid-antitoxin mixtures.

The crude staphylococcus toxoid has three characteristic properties, *viz.*, a lethal-, a necrotic- and a hemolytic power. According to TIMMERMAN (16) the two first mentioned properties are probably linked to one factor, which must be sharply differentiated from the hemolytic property for which evidently another component is accountable. The crude toxoid comprises to all appearance both constituents in the toxoid form. As cited before, the therapeutic effect of the staphylococcus toxoid is as yet far from clear. Apart from this it is quite difficult, if not impossible, to imitate in the animal experiment the human furuncular infections. However in the animal experiment it is quite possible to estimate the antitoxin raising power of the staphylococcal toxoid. On account of the above we limited our experiments for the present to purification by ultrafiltration and we have only once prepared a purified and concentrated staphylococcus toxoid by the salting-out process with ammonium sulfate. With both mentioned purification techniques the total essential protein-complex is completely or nearly so retained in the end product.

EVALUATION OF THE CRUDE AND PURIFIED STAPHYLOCOCCUS TOXOID.

Two methods may be used for the in vitro evaluation. Firstly one can determine the flocculation titre of the crude and the purified (unadsorbed) staphylococcus toxoid, using purified antitoxic staphylococcal serum. In this case the technique of PILLEMER (8) and WITTLER and PILLEMER (17) is applied. Besides the antitoxin-binding method of DOLMAN and KITCHING (4) may be used. For the technique of these methods we refer to the respective articles.

We found, however, that the flocculating and antitoxin-binding power do not parallel in different batches of staphylococcal toxin and toxoid, which is apparent from table 1. According to the technique of DOLMAN and KITCHING the smallest amount

TABLE 1.

Relation between the flocculating titre and antitoxin-binding titre of different batches of staphylococcus toxin and toxoid.

Lot No.	staphylococcus toxin		staphylococcus toxoid		
	Lf/ml	Reciprocal anti-toxin-binding titre	Lot No.	Lf/ml	Reciprocal antitoxin-binding titre
1	9.5	5.3	1	11.5	1.6
2	8.5	4.5	2	8.5	3.6
3	13.–	7.7	3	8.–	4.2
4	13.–	5.3	4	8.–	3.3
5	15.–	10.–	5	10.–	4.2
			6	10.5	8.3
			7	10.–	5.–

of toxin, resp. toxoid, which is capable to combine with one antitoxin unit, is determined. The smaller this amount (expressed in ml) the stronger the toxin, resp. the toxoid.

In order to be able to make a comparison with the flocculating titres (Lf per ml) the reciprocal antitoxin-binding titres are given in the table.

With respect to the purification and concentration of one specific batch of staphylococcus toxoid, a positive correlation however does exist; in other words, the flocculating and antitoxin-binding factors are equally transferred to the end product. So either of the two values may actually be used for checking the progress of purification and concentration. Evaluation of the antigenic power, s.s., of the various crude and purified toxoids, was carried out by means of animal tests, in which the antitoxin contents of the sera of the animals were established as indicated by TIMMERMAN (15). Also the antitoxin contents of the bloodsera of various test persons were estimated conformably.

The culture medium and the strain used.

At first the culture medium as described by PARISH and CLARK (7) was used and their recommendations for incubation were followed. Later on the peptone-Witte solution which they used was replaced by a 3 % solution of Casamino-acids (Difco).

The staphylococcus toxoids prepared by this method possessed the same antigenic activity (animal test) as those prepared by using the ,,old'' nutrient. They could, however, be purified much more easily. All nutrient media were inoculated with the well-known "Wood" strain, originally from Copenhagen, which is always used in our Institute for the preparation of staphylococcus toxin. The staphylococcus toxins, thus obtained, have a M.h.d. = 0.001—0.002 ml and a M.l.d. (for mice) = 0.010—0.020 ml. These toxins were converted into toxoids by adding 0.4% formalin, and heating in the incubator at 37° C. for about one week. For the standard of innocuity which the crude toxoid must meet, we refer to the paper by TIMMERMAN (13) and to the requirements which the Dutch law sets in this regard ¹).

The purified and absorbed toxoids were checked on innocuity

---

¹) Beschikking Min. van Sociale Zaken van 10 Sept. 1935 (Staatsblad no. 176).

by injecting 12 guinea-pigs, of 300 — 400 g body weight, with 5 ml intraperitoneally. None of these animals may show any signs of intoxication during eight weeks after the injection.

The estimation of nitrogen and the purification factor.

Nitrogen was estimated by using the ordinary Kjeldahl method. The purification factor (P.F.) is expressed by the quotient of the flocculation value (resp. the reciprocal antitoxin-binding value) and the nitrogen content of the final product, divided by the corresponding quotient of the starting material.

RESULTS OF THE PURIFICATION EXPERIMENTS.

Only a few typical examples of the performed purifications will be given.

Comparison of total salting-out and ultrafiltration.

1 l of staphylococcus toxoid No. 52, with an antitoxin-binding potency (DOLMAN and KITCHING) = 0.26, and a total N of 0.35 % was salted out by saturating with ammonium sulfate. The precipitate was dissolved in water and dialysed. The final volume was made up to 800 ml and NaCl was added to 0.85 %. The product thus obtained showed the following analytical data:

Antitoxin-binding power = 0.22 (based on 1 l = 0.28).
Total N = 0.077%, which is 18% of the original *N* content (based on 1 l).
Consequently the recovery is 99%, and the P.F. = 6.0.

2 l of this same toxoid were ultrafiltered through an 8 % Parlodion-membrane (Parlodion Mallinckrodt in glacial acetic acid). The final volume was adjusted to 1200 ml and NaCl was added to 0.85 %. This final product showed the following analytical data:

Antitoxin-binding power = 0.24; total N = 0.012%.
Consequently the recovery is 65%, and the P.F. = 27.

On the strength of the fact that ultrafiltration actually gave a smaller yield of antigen but a much superior purification, we used the ultrafiltration technique only in our further experiments. This decision was fully justified by animal experiments which will be described below.

Comparison of purification and concentration, with flocculating and antitoxin-binding power.

32.5 l "casamino-acids"-toxoid no. 67, possessing an antitoxin-binding power of 0.20, a flocculation power = 10 Lf/ml and a nitrogen content of 0.32%, were ultrafiltered through an 8% Parlodion-membrane till a very small volume is left. The ultrafiltrate did not flocculate with antitoxic serum. The residue was made up to 2 l with physiological saline solution, whereupon a jelly-like precipitate was formed, in all probability consisting of agar-agar (see also HOLT (5)). The mixture was filtered through paper and the clear filtrate showed the following analytical data:

Total N = 0.104%, flocculation-titre = 105 Lf/ml.
antitoxin-binding power = 0.18 (dilution 1 : 10).
Consequently: recovery based on flocculating units = 65%.
recovery based on antitoxin-binding power = 69%.
P.F. based on flocculating power = 32.
P.F. based on antitoxin-binding power = 34.
Concentration = 11 times.

In spite of the fact that in these and in other experiments, no toxoid could ever be demonstrated in the ultrafiltrate by a flocculation test, a considerable loss of antigen always occurs.

We have as yet been unable to trace the cause of this phenomenon. It is a well-known fact that the crude staphylococcal toxoid is not very stable for a long time. It is quite possible that this toxoid is partially inactivated or adsorbed on the collodium-membrane during ultrafiltration.

HOLT (5) too mentions the comparative instability of the crude product.

ANIMAL TESTS.

In the first place it was our aim to find out whether the toxoid made by means of the "casamino-acids" medium was equal in antigenic power to the staphylococcus toxoid made by means of the "old" medium of PARISH and CLARK (7). Apart from this we wanted to gather information on the immunizing capacity of the purified (unadsorbed) toxoid, compared in this respect with the crude product, and finally we wanted to answer the questions to what extent the adsorption of the purified antigen on aluminium phosphate enhances the immunizing capacity.

To this end, five groups of guinea-pigs, comprising 16 animals each, were treated in the undermentioned way.

Group 1 was given three subcutaneous injections of 0.5, 1.0 and 1 ml, resp. of crude toxoid (10 Lf/ml), prepared with the "old" medium of Parish and Clark with an interval of two weeks between the injections. Four weeks after the final injections the animals were bled and the antitoxin titres of their sera were determined.

Group 2 received the same treatment as group 1. The toxoid (10.5 Lf/ml) however was derived from the "casamino-acids" medium.

Group 3 was treated in the same manner as group 2. The toxoid prepared via the "casamino-acids" medium was however purified and subsequently diluted to the original strength (10.5 Lf/ml).

Group 4 received 2 intramuscular injections of 0.5 ml purified "casamino-acids"-toxoid, original strength (as group 3) but adsorbed on 10 mg $AlPO_4$ per ml. The interval between the injections was 4 weeks. 4 weeks after the last injection the animals were bled and their sera were titrated.

Group 5 got a similar treatment as group 4, the purified and adsorbed staphylococcus toxoid was, however, concentrated twofold (21 Lf/ml and the identical $AlPO_4$-concentration = 10 mg per ml). The results of these experiments are given in table 2.

TABLE 2.

Difference in antigenic power between various crude, purified and adsorbed staphylococcus toxoids.

Group	Titres of the separate guinea-pig sera in AU/ml																Mean titre AU/ml	σ^2
1	0.2	0.5	<0.2	0.2	0.2	0.2	3.0	<0.2	0.2	0.2	0.2	1.0	0.5	<0.2	0.2	2.–	0.54	0.636
2	0.2	1.0	0.2	0.5	0.3	<0.2	0.2	0.5	1.0	0.2	<0.2	0.2	1.0	2.0	1.0	1.0	0.56	0.300
3	0.2	1.5	1.0	2.0	0.5	1.0	<0.2	<0.2	5.0	0.2	<0.2	0.5	0.2	0.5	0.3	0.2	0.81	1.48
4	3.0	3.0	2.0	1.5	3.0	0.2	1.0	2.0	0.5	1.0	1.0	0.2	3.0	1.0	10.0	0.5	2.6	2.67
5	9.0	8.0	1.0	0.2	2.0	4.0	2.0	2.0	3.0	15.0	1.0	3.0	1.0	2.0	2.0	2.0	3.7	13.5

Difference between the titres of group 2 and group 1 = 0.02.
Standard error = 0.24. Difference is not significant.
Difference between the titres of group 3 and group 2 = 0.25.
Standard error = 0.33. Difference is not significant.
Difference between the titres of group 4 and group 2 = 2.04.
Standard error = 0.44. Difference is significant.
Difference between the titres of group 5 and group 4 = 1.1.
Standard error = 1.00. Difference is not significant.

Based on the above described experiments we come to the following conclusions.

1) The crude staphylococcus toxoid derived from the casamino-acids medium (group 2), immunizes just as well as the toxoid derived from the "old" medium of PARISH and CLARK (group 1).
2) The purified staphylococcus toxoid immunizes at least as well as the crude product (group 3 and 2).
3) The purified and adsorbed staphylococcus toxoid (group 4) immunizes much better than the unadsorbed toxoid (group 3).
4) The concentration of the purified and adsorbed product has, within certain limits and considering our small groups of test animals only little or no influence on the immunizing capacity of this preparation (groups 4 and 5).

Furthermore we have tried to ascertain to what extent the number of injections and the interval between the injections, resp. between the final injection and the bleeding of the animals, would affect the antitoxin production.

To this purpose four groups of 12 guinea-pigs each were treated in the following manner.

Group 1 received 2 intramuscular injections of 0.5 ml staphylococcus-P.T. (prepared from a "casamino-acids" medium, 10.5 Lf/ml and 10 mg $A1PO_4$ per ml), with an interval of one week, while 2 weeks after the second injection the animals were bled and their antitoxin titres determined.

Group 2 received 3 intramuscular injections of 0.5 ml of the same staphylococcal-P.T. with intervals of one week. Bleeding and serum-titration were carried out 2 weeks after the final injection.

Group 3 received 2 intramuscular injections with P.T. of 0.5 ml each spaced at 4 weeks. Bleeding and serum-titration were carried out 4 weeks after the final injection.

Group 4 received 2 injections of 0.5 ml each of staphylococcal-P.T. with an interval of one week, while bleeding and serum-titration were carried out one week after the final injection. The results of these experiments are summarized in table 3.

On closer inspection of these experiments, we come to the conclusion that the number of injections and the intervals between these and between the last injection and determination of the serum titres, affect the results of our experiments only slightly.

TABLE 3.

Influence of the number of staphylococcal-P.T. injections, the interval between the injections and the number of weeks between the last injection and the bleeding of the animals, on the amount of staphylococcal-antitoxin formed.

Group	Titres of the separate guinea-pig sera in AU/ml												Mean titre AU/ml
1	8.0	3.0	3.0	2.0	4.0	0.2	4.0	2.0	1.5	10.0	2.0	4.0	3.6
2	3.0	4.0	2.0	1.0	4.0	1.5	2.0	2.0	1.5	5.0	2.0	5.0	2.5
3	4.0	7.0	5.0	1.0	1.0	2.0	2.0	3.0	8.0	1.0	1.5	—	3.2
4	8.0	3.0	5.0	1.0	<0.2	2.0	9.0	1.0	1.0	<0.2	2.0	1.0	2.6

EXPERIMENTS ON HUMAN BEINGS.

On the strength of the information gained from above mentioned animal tests, we expected good immunizing properties of the staphylococcal-P.T. when used in man.

Thirty volunteers, most of them belonging to the staff of our Institute, were treated with staphylococcal-P.T. (10.5 Lf/ml and 10 mg $AlPO_4$ per ml). Before the first injection with the antigen the titre of circulating antitoxin in their blood was estimated.

Besides they were asked whether they had received any previous treatment with staphylococcus toxoid or staphylococcal vaccine. Hereupon they received 2 intramuscular injections (0.1 ml) of staphylococcal-P.T. with a spacing of one week. Two weeks after the final injection their antitoxin content was again determined in the serum. This last blood specimen was also tested on agglutinins against the "Wood" strain with which the original staphylococcus toxoid had been prepared. Moreover we tried to agglutinize with the various serum-samples other staphylococcal strains derived from patients. In these cases we investigated, these agglutination tests yielded negative results.

Lastly the local and general reactions were established after every injection. The quantitative results are compiled in table 4.

These findings demonstrate that the purified and adsorbed staphylococcus toxoid actually is a very good antigen.

With the exception of three out of thirty (Nos 7, 16 and 22) all treated test persons showed a marked increase in their antitoxin-titre. Considering that only two injections of 0.1 ml each of staphylococcal-P.T. were given with a spacing of only one week, and that the antitoxin titration took place only two weeks after the final injection, the results of these experiments may be called remarkable.

TABLE 4.

Antitoxin-titres of test persons before and after immunization with two injections of 0.1 ml staphylococcal-P.T.

No. test person	Titre *before* immunization (AU/ml)	Titre *after* immunization (AU/ml)	Previous treatment with staphylococcal toxoid or vaccine
1	2.0	9.0	toxoid and vaccine
2	0.75	8.0	no
3	0.20	5.0	no
4	2.5	4.5	toxoid and vaccine
5	0.50	3.5	no
6	0.75	1.5	toxoid and vaccine
7	0.50	0.50	no
8	0.20	1.0	no
9	0.20	1.0	no
10	0.20	3.0	no
11	1.0	8.0	no
12	0.20	0.50	no
13	2.0	3.5	vaccine
14	0.20	9.0	no
15	0.50	7.0	no
16	1.5	1.5	no
17	0.75	2.5	no
18	6.0	10.0	no
19	0.20	5.5	no
20	1.0	1.5	no
21	1.5	19.0	no
22	0.50	0.50	no
23	0.50	7.0	no
24	1.0	11.0	no
25	0.50	7.0	no
26	0.20	1.5	no
27	1.0	7.0	no
28	0.50	2.0	no
29	0.20	6.0	no
30	0.20	3.0	no

We could not observe any connection between the increase in titre and a previous treatment with either staphylococcus toxoid or vaccine.

As for local and general reactions, the following may be pointed out. With a few exceptions all test persons declared that the injections caused some inconvenience which usually manifested itself in the form of a slight soreness and stiffness of the arm into which the injection had been given. Besides some of them complained of a slight general reaction. After 48 hours however there were no more complaints. Some of these test persons had previously volunteered in an investigation on the application of diphtheria-P.T. (Noordam, Brandwijk, Tasman and Timmerman (6)). They declared that they thought the reaction, following the two injections with 0.1 ml staphylococcus toxoid, stronger than the one upon two injections of 0.5 ml diphtheria-P.T. The reactions after the second staphylococcus-P.T. injection were stronger than those after the first one. Infiltration or redness on or around the injection-site were not observed. For about one to two days the injection-site was however slightly to distinctly painful upon pressure.

None of the test persons absented from work on account of the injections. Naturally the appreciation of the reactions by the test persons is highly subjective.

We think it however permissible to conclude that we succeeded in preparing a staphylococcal antigen which causes considerably less unpleasant local and general reactions than the crude and unadsorbed staphylococcus toxoid.

Furthermore we arrive at the conclusion that purified staphylococcus toxoid, adsorbed on aluminium phosphate, is a very active antigen. Although we think that the local and general reactions would not raise any serious objections to the therapeutic application of this novel preparation, it would pay to investigate how far it would be possible to further purify the ultrafiltered and adsorbed staphylococcus toxoid, with conservation of the antigenic properties. Perhaps it would then be possible to prepare an active antigen which causes few or no reactions.

Summary.

1. The potency of crude or purified staphylococcus toxoid may be estimated in vitro by two methods, *viz.*, by the flocculation method (Pillemer and Wittler and Pillemer), and by the antitoxin-binding method (Dolman and Kitching). The flocculation values and the antitoxin-binding values appear not to correlate for different batches of staphylococcus toxin and toxoid. There is good correlation however during the purification of one specific batch. So either value may be used for checking the progress of purification and concentration.

2. At first the formulae of PARISH and CLARK concerning the medium from which the staphylococcus toxin (resp. toxoid) was derived, were followed. Later on the indicated peptone-Witte solution was successfully replaced by a 3 % solution of casamino-acids (Difco). The toxoids thus prepared were much easier to purify than the staphylococcus toxoid prepared according to the ,,old" formulae.

3. The purification of the staphylococcus toxoid was carried out by using the total salting-out process with ammonium sulfate (P.F. = 6.0, recovery 99 %) as well as by ultrafiltration through an 8 % Parlodion-membrane (P.F. = 27, recovery 65 %). On the strength of this experience all subsequent purifications were performed employing the ultrafiltration technique.

4. In animal experiments the purified staphylococcus toxoid appeared to immunize at least as well as the crude product.

5. The antigenicity could be considerably enhanced by adsorption of the purified staphylococcus toxoid on aluminium phosphate. The number of flocculation units per ml of the purified and adsorbed toxoid appeared to have, within certain limits, no significant influence on the immunizing capacity of the preparation.

6. The number of injections, their spacing and the interval between the final injection and the estimation of the serum-titre of the animals, affect the experimental results only slightly too.

7. Thirty volunteers were given two injections of 0.1 ml each of purified and adsorbed staphylococcus toxoid (10.5 Lf/ml and 10 mg $AlPO_4$ per ml) with an interval of one week. Two weeks after the final injection 27 out of 30 test persons showed an appreciable or very strong raise of their antitoxin titre. Any connection with a previous treatment with crude staphylococcus toxoid or staphylococcus vaccine could not be established.

8. In the course of these immunizations no agglutinines against staphylococci (the "Wood"-strain with which the toxin had been prepared, or with strains derived from patients) were formed.

9. There were only few local and general reactions and none after 48 hours. Absence from work on account of the injections did not occur.

10. It was proved to be possible to prepare a staphylococcal antigen which owing to the purification and adsorption on aluminium phosphate, possesses a very satisfactory immunizing potency, while the inoculation-reactions are much weaker than those usually caused by the crude staphylococcus toxoid.

Literature.

1. A. Boivin and Y. Izard, C. R. Soc. Biol. **124,** 25, 1937. – 2. F. M. Burnet J. Pathol. Bact. **33,** 1, 1930; **34,** 471, 759, 1931. – 3. F. M. Burnet and M. Freeman, J. Pathol. Bact. **35,** 477, 1932. – 4. C. E. Dolman and J. S. Kitching, J. Pathol. Bact. **41,** 137, 1935. – 5. L. B. Holt, Brit. J. Exp. Pathol. **17,** 318, 1936. – 6. A. L. Noordam, A. C. Brandwijk, A. Tasman and W. Aeg. Timmerman, Ned. Tijdschr. v. Geneesk. **93,** 4014, 1949. – 7. H. J. Parish and W. H. M. Clark, J. Pathol. Bact. **34,** 593, 1931; **35,** 251, 1932. – 8. L. Pillemer, J. Immunol. **53,** 237, 1946. – 9. G. Ramon, C. R. Soc. Biol. **121,** 375, 1936. – 10. G. Ramon, R. Richou et M. Djourichitch, Revue Immunol. **2,** 482, 1936. – 11. J. D. van Ramshorst, De bereiding van diphtherie entstoffen. Thesis Utrecht, 1951, p. 60. – 12. W. Aeg. Timmerman, Ned. Tijdschr. Hyg. Microb. en Serol. **7,** 178, 1933. – 13. W. Aeg. Timmerman, Antonie van Leeuwenhoek **2,** 377, 1935. – 14. W. Aeg. Timmerman, Ned. Tijdschr. v. Geneesk. **81,** 1725, 1937. – 15. W. Aeg. Timmerman, Brit. J. Exp. Pathol. **18,** 406, 1937. – 16. W. Aeg. Timmerman, Antonie van Leeuwenhoek **8,** 41, 1942. – 17. R. G. Wittler and L. Pillemer, J. Biol. Chem. **174,** 23, 1948.

(Aus dem Bakteriologischen Laboratorium und dem Pathologisch-Anatomischen Laboratorium zu Paramaribo, Suriname).

BEITRÄGE ZUR GEOGRAPHISCHEN PATHOLOGIE VON SURINAME [1]

6. HISTOPLASMOSE BEI SÄUGETIEREN IN SURINAME

von

W. A. COLLIER und **W. E. F. WINCKEL**

(Empfangen 24 April 1952).

Die Epidemiologie der Histoplasmose stellt uns noch immer vor sehr grosse Rätsel. Die Krankheit scheint nicht kontagiös zu sein, denn weder Beobachtungen beim Menschen noch Tierversuche weisen auf Infectionen durch Contact.

Schon DARLING (5) untersuchte verschiedene Tierspecies in Panama, da er damals schon an Reservoire dachte, konnte aber in deren Faeces keine Parasiten nachweisen. Seitdem hat man aber bei verschiedenen Säugetieren in den Vereinigten Staaten Histoplasmen gefunden, und manche Untersucher neigen zu der Ansicht, dass infizierte Säuger möglicherweise eine gewisse Rolle bei der Epidemiologie der Histoplasmose spielen dürften.

Dem steht die vor kurzer Zeit ausgesprochene Theorie von BEADENKOPF und LOOSLI (1) gegenüber, die an eine ausschliesslich durch die Luft verbreitete Infection denken. Sie nehmen an, dass die mit dem Schlamm vermengten Pilzmassen durch die Ueberschwemmungen des Mississippi weithin über das Land verbreitet werden, nach Zurückfluten des Wassers austrocknen und schliesslich durch den Wind weithin verschleppt werden. Die hohen Prozentsätze der histoplasmin-positiven Personen in den Staaten des Mittelwestens (bis zu 95%!) machen zwar eine Masseninfection durchaus wahrscheinlich, fraglich bleibt indessen, wo sich diese hierzu erforderlichen grossen Mengen von Infections-Stoff entwickeln sollten. Im Mississippi-Schlamm müssten gewaltige Brutnester von Histoplasma nachweisbar sein, um nach Verstäubung des getrockneten Schlammes genügend Material hervorzubringen, das praktisch die gesammte Bevölkerung ganzer Staaten zu infizieren vermag.

---

[1] 1. Mitt.: W. A. COLLIER, A. E. WOLFF und A. E. G. ZAAL, Documenta Med. Geogr. et Trop. **4**, 92, 1952; 2. Mitt.: W. A. COLLIER und W. E. F. WINCKEL, Z. f. Hyg. im Druck; 3. Mitt.: C. F. A. BRUYNING, Documenta Med. Geogr. et Trop. **4**, 171, 1952; 4. Mitt.: W. A. COLLIER und D. A. DE LA PARRA, Documenta Med. Geogr. et Trop. im Druck; 5. Mitt.: W. E. F. WINCKEL, W. A. COLLIER und A. E. G. TREURNIET, Documenta Med. Geogr. et Trop. im Druck.

Weitherhin kommt aber Histoplasmose auch in Gegenden vor, wo dergleichen Ueberschwemmungen nicht zu beobachten sind. In Paramaribo in Suriname ist letzteres beispielsweise nicht der Fall, und der hier fast stets von der See her wehende Wind kann schwerlich verantwortlich gemacht werden, Histoplasma in die Stadt zu bringen. Trotzdem scheint Histoplasmose in Paramaribo nicht selten zu sein, wurden doch nach Angabe von WINCKEL, COLLIER und TREURNIET (26) im Jahre 1951 durch Probe-Excisionen und im Tierversuch 45 Fälle menschlicher Histoplasmose nachgewiesen. Weiterhin wurde durch F. PHENNING in 1952 laut mündlicher Mitteilung an einer Stelle der Stadt (Wolffenbuttel) beobachtet, dass die Histoplasminreaction bei einer kleinen Gruppe von Personen zu 50% positiv war.

Während eine vorwiegend durch die Luft erfolgte Infection in Paramaribo nicht sehr wahrscheinlich ist, möchte man hier mehr an tierische Reservoire denken, von denen aus die menschlichen Infectionen ihren Ausgang nehmen. Nachdem verschiedene Vögel aus Paramaribo und Umgebung durch COLLIER und WINCKEL (4) untersucht worden waren und den Nachweis von spontaner Histoplasmose ermöglicht hatten, wurde weiterhin bei Säugetieren auf Histoplasmeninfectionen gefahndet.

Die Milz der Tiere wurde durchschnitten, und von der Schnittfläche wurden Tupf-Präparate hergestellt, die nach Giemsa gefärbt wurden. Von einem Teil der Tiere wurde auch Milzsuspension auf weisse Mäuse verimpft. Diese wurden nach Ablauf von 3 Wochen getötet, worauf die Milz in gleicher Weise untersucht wurde. Kulturen konnten aus äusseren Gründen bisher noch nicht angelegt werden. Als positiv wurden die Tiere bezeichnet, die in der Milz intracellulär und öfters auch extracellulär auf Histoplasma gleichende Gebilde erkennen liessen, wobei nicht berücksichtigt wurde, ob nicht neben Histoplasma capsulatum noch andere Histoplasma-Formen in Frage kommen könnten.

Die Resultate wurden in Tabelle 1 eingetragen, worin ausserdem vergleichsweise die uns erreichbaren bisherigen Befunde anderer Autoren bei Säugetieren aufgenommen sind.

TABELLE 1.

Uebersicht über die bisherigen Befunde von Spontan-Infectionen mit Histoplasma bei Säugetieren.

Tierspecies	Land	Zahl	Positiv	Literatur
Marsupialia				
„marsupials"	Panama		0	(5)
Didelphis virginiana	Virginia	3	0	(10)
	Virginia	1	1	(8)
Didelphis virg. pigra	Georgia	1	0	(11)
Metachirus nudicaudatus	**Suriname**	5	3	
Insectivora				
Blarina brevicaudata	Virginia	76	0	(10)
Chiroptera				
Phyllostomus hastatus panamensis	Panama		0	(25)
Hemiderma perspicillatum aztecum	Panama		0	(25)
Desmodus rotundus murinus	Panama		0	(25)
„Fledermäuse" (indeterm.)	**Suriname**	27	2	
Primates				
„monkeys"	Panama		0	(5)
Alouatta senicula	**Suriname**	1	1	
Cebus fatuellus trepidus	**Suriname**	1	0	
? *Callicebus ornatus*	**Suriname**	2	2=immun	
Saimiri sciureus	**Suriname**	2	2=immun	
	Suriname	1	0	
Edentata				
Cyclopes didactylus	**Suriname**	1	1	
Choloepus didactylus	**Suriname**	1	1	
Dasypus novemcinctus	**Suriname**	1	0	
Lagomorpha				
Sylvilagus auduboni	Texas	10	0	(8)
Sylvilagus floridanus	Virginia	42	0	(10)
	Georgia	2	0	(11)
Rodentia				
Sciurus carolinensis	Georgia	1	0	(11)
Sciurus niger niger	Georgia	2	0	(11)
Marmota monax	Virginia	13	0	(10)
Cynomys ludovicianus	Texas	6	0	(8)

Tierspecies	Land	Zahl	Positiv	Literatur
Citellus mexicanus	Texas	6	0	(8)
Citellus spilosoma	Texas	11	0	(8)
Citellus tridecimlineatus	Texas	1	0	(8)
Dipodomys ordii	Texas	32	0	(8)
Dipodomys spectabilis	Texas	16	0	(8)
Peromyscus spec.	Virginia	101	0	(10)
Peromyscus gossypinus	Georgia	6	0	(11)
Onychomys leucogaster	Texas	17	0	(8)
Sigmodon hispidus	Georgia	41	0	(11)
Neotoma micropus	Texas	617	0	(8)
Pitymys pinetorum	Virginia	2	0	(10)
Microtus spec.	Virginia	196	0	(10)
Zapus hudsonius	Virginia	1	0	(10)
„rats"	Panama		0	(5)
	USA.		+	(12)
Rattus norvegicus	Virginia	222	5	(10)
	Virginia	565	16	(9)
	Virginia		27	(11)
	Georgia	459	7	(11)
	U.S.A.		+	(7)
	Texas	9	0	(8)
	Virginia + Georgia		47	(8)
	Baltimore	629	0	(8)
	Maryland	64	0	(8)
	Texas	744	0	(8)
	Suriname	6	0	
R. norvegicus Albino	India		1	(22)
Rattus r. alexandrinus	Georgia	855	4	(11)
	Virginia + Georgia		4	(8)
	Suriname	46	2	
Rattus r. frugivorus	**Suriname**	1	1	
? *Rattus* spec.	**Suriname**	12	0	
Mus musculus	Panama		0	(5)
	U.S.A.		+	(16)
	U.S.A.		+	(23)
	Virginia		1	(17)
	Virginia	936	1	(10)
	Virginia		1	(9)
	Georgia	14	0	(11)
	Virginia + Georgia		1	(8)
	Suriname	2	0	

Tierspecies	Land	Zahl	positiv	Literatur
Mus musculus albino	Italien		+	(20)
	India		+	(22)
	Suriname	46	0	
Carnivora				
Canis familiaris	Tennessee		1	(16)
	U.S.A.		1	(24)
	St. Louis		1	(3)
	U.S.A.		2	(2)
	Panama		1	(25)
	Brasilien		1	(18)
	U.S.A.		1	(21)
	Virginia	7	0	(10)
	Virginia		3	(17)
	U.S.A.		+	(6)
	U.S.A.		2	(13)
	U.S.A.		+	(7)
	U.S.A.		4	(19)
	U.S.A.	837	Test = 5	(19)
	Virginia + Georgia		5	(8)
	Suriname	81	5	
	Suriname	27	via Maus = 9	
Vulpes spec. + *Urocyon* spec.	Virginia	7	0	(10)
Urocyon cinereoargenteus floridanus	Georgia	2	0	(11)
Cerdocyon thous thous	**Suriname**	1	0	(5)
,,racoons''	Panama		0	(5)
Procyon lotor	Virginia	5	0	(10)
Procyon lotor elucus	Georgia	1	0	(11)
Mustela spec.	Virginia	4	0	(10)
Putorius furo	U.S.A.		1	(14)
Spilogale putorius	U.S.A.		+	(7)
	Georgia	6	5	(11)
	Virginia + Georgia		7	(8)
Felis catus	Virginia	5	0	(10)
	Georgia	1	0	(11)
	U.S.A.		+	(7)
	Virginia + Georgia		19	(8)

Tierspecies	Land	Zahl	positiv	Literatur
Artiodactyla				
Ovis aries	**Suriname**	1	0	
Bos taurus	Panama		0	(5)
	Suriname	16	1	
Sus scrofa	**Suriname**	11	0	

Von Marsupialien wurden 5 *Metachirus nudicaudatus* untersucht, von denen die 3 ausgewachsenen Tiere alle Splenomegalie aufwiesen und positiv waren. Von den untersuchten 27 Chiropteren waren 2 Tiere positiv, doch konnten die Tiere bisher noch nicht determiniert werden.

Der positive *Alouatta senicula* (Brüllaffe) stammte aus der weiteren Umgebung von Paramaribo und machte einen deutlich kränklichen Eindruck. Die Milz des Tieres war ausserordentlich stark geschwollen und wies besonders viel intra- und extracellulär liegende auf Histoplasma gleichende Parasiten auf.

Je 2 *Callicebus ornatus* und *Saimiri sciureus* wurden intraperitoneal mit sehr grossen Dosen von histoplasmahaltiger Mäusemilz infiziert und 3—4 Wochen später getötet. Bei der Section zeigten sich keine specifischen Abweichungen und liessen sich keine auf Histoplasma gleichende Parasiten nachweisen. Ueber die Dauer einer Immunität nach Infectionen mit Histoplasma ist bisher sehr wenig bekannt. Es lässt sich daher nicht angeben, ob es sich bei diesen Tieren um eine Immunität nach überstandener Infection im Wildzustand gehandelt hat, sodass man an eine Analogie mit gelbfieber-immunen Affen in Gelbfiebergebieten denken könnte. De Monbreun (15) hatte Affen als sehr empfänglich gefunden.

Von den drei untersuchten Edentaten wies *Cyclopes didactylus* (kleiner Ameisenesser) starke Splenomegalie auf. Dieses Tier und *Choloepus didactylus* (grosses Faultier) waren beide positiv, während *Dasypus novemcinctus* (Gürteltier, „tatú") negativ war. Letztere Species ist ein Erdbewohner, im Gegensatz zu den beiden erstgenannten Edentaten, die Strauch- bezw. Baumbewohner sind.

Von Nagern wurden 6 *R. norvegicus* untersucht, die alle negativ waren, während 2 von 46 *Rattus rattus alexandrinus* positiv waren. Dies war auch der Fall bei einer *Rattus rattus frugivorus* vom Rande der Stadt. Weiterhin waren 12 nicht näher determinierte rattenähnliche Nager negativ, ebenso 2 *Mus musculus*. Bei 46 weissen Mäusen (Controlltiere) liessen sich gleichfalls keine Parasiten nachweisen.

Interessant ist das Ergebnis der Untersuchung von Hunden. Von einem Tier, einer Bastard-Hündin von ca. 8 Jahren, die aus äusseren Gründen getötet werden musste, wurden Stücke von Leber, Milz und einige Knötchen von Haut und Vulva zur Untersuchung eingesandt. Die Haut des Tieres war mit diesen Knötchen besät, die in Cutis und Subcutis lagen. In Leber und Milz fanden sich haselnussgrosse bis kastaniengrosse Knoten, die zich mässig hart anfühlten und im Durchschnitt eine fleckig-grauweisse Farbe aufwiesen. Microscopisch handelte es sich um Geschwülste mit vorwiegend expansivem und nur gelegentlich infiltrativem Wachstum von trabekulärem Bau mit vielen Capillaren. Der Celltypus war rund (granulocytengross) mit grossen, runden, mässig chromatinreichen Kernen. Es fanden sich zahlreiche Mitosen. Das Ganze machte den Eindruck von metastatisch verbreiteten Tumoren epithelialen Ursprungs. Da nur einzelne Organstücke eingesandt worden waren, kann über einen Primärtumor nichts ausgesagt werden. In der Leber zeigte sich ausgedehnte Amyloidosis. Die Milz wies das Bild einer subchronischen Splenitis auf.In den Reticulumcellen der Milz und in einzelnen Lebercellen wurden Einschlüsse gefunden, die von Histoplasma nicht zu unterscheiden waren. In den Geschwulstcellen wurden diese Einschlüsse nicht nachgewiesen.

Im Ganzen wurden von 81 Hunden (einschliesslich des oben erwähnten) die Milz untersucht. Von diesen wiesen 5 die auf Histoplasma gleichenden Einschlüsse auf. Weiterhin aber wurde die Milz von 27 Hunden (von diesen 81) auf weisse Mäuse verimpft. Nach 3 Wochen wurden die Tiere getötet, und bei der Untersuchung ihrer Milz ergab es sich, dass in 9 Fällen auf Histoplasma gleichende Einschlüsse nachweisbar waren.

In einem Fall wurden auch in der Milz eines geschlachteten Rindes auf Histoplasma gleichende Gebilde nachgewisen.

Ebenso wie in den Vereinigten Staaten gelang es also auch in Suriname, bei verschiedenen wildlebenden Säugetieren und auch bei Haustieren auf Histoplasma gleichende Gebilde nachzuweisen. Da es ferner Collier und Winckel (4) geglückt war, auch bei Haus- und Wildvögeln derartige, auch für weisse Mäuse pathogene Formen, nachzuweisen, so darf man wohl annehmen, dass in Suriname bei warmblütigen Wirbeltieren die Infectionen mit Histoplasmen nicht zu den Seltenheiten gehören. Möglicherweise spielen diese Tiere die Rolle von Reservoiren, von denen aus der Mensch infiziert werden kann, vielleicht auch sind sie nur die Endglieder von Infect-ketten.

Nach noch nicht veröffentlichten Versuchen von Collier und Bruining ist es nicht ausgeschlossen, dass hierbei bestimmte blutsaugende Arthropoden eine Rolle spielen.

Zusammenfassung.

Bei einer Reihe von Säugetieren in Suriname fanden sich in der Milz bei der Untersuchung von Tupfpräparaten intracellulär und gelegentlich auch extracellulär gelagerte Gebilde, die von Histoplasma nicht zu unterscheiden waren. In einer Reihe von Fällen liessen sich die Parasiten auf weisse Mäuse verimpfen und bei diesen gleichfalls in der Milz zurückfinden.

Literatur.

1. W. G. Beadenkopf and C. G. Loosli, J. Amer. Med. Ass. **146, 621,** 1951. – 2. R. F. Birge and W. H. Riser, North Americ. Vet. **26,** 281, 1945. – 3. W. P. Callahan, Amer. J. Trop. Med. **24,** 363, 1944. – 4. W. A. Collier und W. E. F. Winckel, Z. f. Hyg. (im Druck) 1952. – 5. S. T. Darling, Arch. Intern. Med. **2,** 107, 1908. – 6. J. T. Duncan, Trop. Dis. Bull. **45,** 207, 1948. – 7. C. W. Emmons, Trans. New York Acad. Sci. **11,** 248, 1949. – 8. C. W. Emmons, Amer. J. Publ. Health **40,** 436, 1950. – 9. C. W. Emmons and L. L. Asburn, Publ. Health Rep. **63,** 1416, 1948. – 10. C. W. Emmons, J. A. Bell and B. J. Oslon, Publ. Health Rep. **62,** 1642, 1947. – 11. C. W. Emmons, H. B. Morlan and E. L. Hill, Publ. Health Rep. **64,** 1423, 1949. – 12. G. H. Hansmann and J. R. Schenken, Amer. J. Path. **10,** 731, 1934. – 13. K. S. Harmon, J. Amer. Vet. Med. Ass. **113,** 60, 1948, – 14. N. D. Levine, G. L. Dunlap and R. Graham, Cornell Veter. **28,** 249, 1938. – 15. W. A. de Monbreun, Amer. J. Trop. Med. **14,** 93, 1934. – 16. W. A. de Monbreun, Amer. J. Trop. Med. **19,** 565, 1939. – 17. B. J. Olson, J. A. Bell and C. W. Emmons, Amer. J. Publ. Health **37,** 441, 1947. – 18. M. Para, Amer. J. Trop. Med. **26,** 273, 1946. – 19. J. A. Prior, C. R. Cole and V. Torbet, Publ. Health Rep. **64,** 1562, 1949. – 20. G. Sangiorgi, Pathologica **14,** 493, 1922. – 21. H. R. Seibold, J. Amer. Vet. Med. Ass. **109,** 209, 1946. – 22. H. E. Shortt, Ind. J. Med. Res. **10,** 908, 1923. – 23. M. Tager and A. A. Liebow, Yale J. Biol. a. Med. **14,** 469, 1942. – 24. J. M. Thuringer, Arch. of Path. **37,** 140, 1944. – 25. W. J. Tomlinson and R. G. Grocott, Amer. J. Clin. Path. **15,** 501, 1945. – 26. W. E. F. Winckel, W. A. Collier und A. E. G. Treurniet, Documenta Neerl. (im Druck) 1952.

(From the National Institute for Public Health, Utrecht, Holland).

ON THE PREPARATION AND PROPERTIES OF A NEW TETANUS-PROPHYLACTIC, THE "TETANUS-P.T."

by

A. TASMAN and **J. D. VAN RAMSHORST**

(Received June 23, 1952).

In view of the excellent results (3, 6) obtained with the application of diphtheria-phosphate-toxoid (Diphtherie-P.T.), an attempt was made to purify crude tetanus toxoid and to raise the antigenicity of the purified product by adsorption on aluminium phosphate.

For the purification and concentration of the crude tetanus toxoid a technique identical to that used for diphtheria toxoid (7) was employed, i.e. ultrafiltration and salting-out with ammonium sulfate.

Quantitative estimation in vitro of the tetanus toxoid was performed by means of the flocculation-reaction of RAMON, carried out as described by MOLONEY and HENNESY (5) and by LEVINE and STONE (4).

The purification progress was checked by comparing the antigen titre found via the flocculation-reaction and the content of total N, resp. protein-N as determined by the KJELDAHL method. The quotient of the flocculation titre per mg total N of the final product, and the corresponding value for the starting material is indicated as "Purification factor" (P.F.). So the degree of purification achieved is expressed as the ratio

$$\text{P. F.} = \frac{\text{Lf/mg total N final product}}{\text{Lf/mg total N starting material}}$$

The recovery was calculated in proportion to the total amount of flocculation units present in the starting material.

As stated above, the purification process comprises two steps, i.e. ultrafiltration and ammonium sulfate fractionation. By ultrafiltration (through an 8% Parlodion-membrane) a substantial part of the "non-protein" nitrogen components, electrolytes and sugars is eliminated together with a small portion of the proteins. By this method actually very little purification with respect to protein nitrogen is obtained. The main purpose of the ultrafiltration however is a concentration of the antigen in the residue.

This residue was subsequently subjected to purification by ammonium sulfate fractionation. Before applying this treatment to the bulk of the ultrafiltration residue the proper salt concentrations were established in a preliminary test, conformable to the procedure applied for the purification of diphtheria toxoid (VAN RAMSHORST (7) and BRANDWIJK and TASMAN (1, 2)).

TABLE 1.

Fractionation of ultrafiltered tetanus toxoid by means of ammonium sulfate.

% $(NH_4)_2SO_4$	Percent of the toxoid precipitated						
	Toxoid 495	Toxoid 498	Mixture P.T. 1	Mixture P.T. 2	Mixture P.T. 3	Mixture P.T. 4	Mixture P.T. 5
15.0	—	—	—	—	—	0	—
16.0	—	—	—	35	—	—	0
17.5	—	0	—	—	0	100	—
18.0	—	—	—	90	—	—	53
20.0	—	50	—	100	14	100	90
22.0	—	—	—	—	—	—	98
22.5	30	94	70	100	68	100	—
25.0	—	96	75	100	84	—	—
27.5	85	100	85	—	—	—	—
30.0	92	100	91	—	100	—	—
32.5	100	100	—	—	—	—	—

In table 1 data concerning some of these fractionations are summarized of which a few are interpreted graphically in fig. 1.

When these salting-out curves are compared with similar curves of diphtheria toxoid (BRANDWIJK and TASMAN (1, 2)) the following may be pointed out.

1. The curves of tetanus toxoid are much steeper than the corresponding diphtheria toxoid curves. In other words, the tetanus antigen is linked to a protein fraction which can be precipitated within a very small concentration-zone with ammonium sulfate. In this connection it may be well to remark that, prior to the

antigen precipitation, practically no other proteins are precipitated by the ammonium sulfate. This implies in practice that the ultrafiltered tetanus toxoid can be precipitated at one definite ammonium sulfate concentration. This is in contrast with the diphtheria toxoid purification which comprises a two-step fractionation process with ammonium sulfate (i.e. the desired protein fraction may be precipitated between two clearly defined ammonium sulfate concentrations).

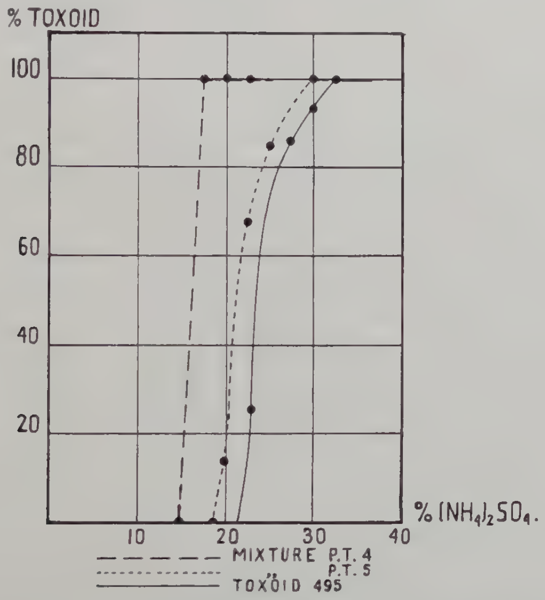


Fig. 1. Fractionation of ultrafiltered tetanus toxoid by means of ammonium sulfate.

2. Conformable to the fractionation of different batches of diphtheria toxoid, the various tetanus toxoid batches show different salting-out curves. The practical consequence of this is that, as with diphtheria toxoid, the right ammonium sulfate concentration must be established experimentally, prior to every tetanus toxoid purification.

Basing our technique on previous experience, we purified different batches of tetanus toxoid by ultrafiltration and ammonium sulfate fractionation. The quantitative results are summarized in table 2. The flocculation titres of the various products (Lf/ml) are referred to the new International Tetanus Antitoxin standard.

Purification of different ba

CRUDE PRODUCT						ULTRAFILTRA			
Toxoid No.	Lf/ ml	% total N	% P.N.	Lf/ mg total N	Lf/ mg P.N.	Lf/ ml	% re-covery	% total N	P.
495	5.–	0.660	0.0126	0.75	40	9	90	0.051	0.0
498	5.6	0.618	0.052	0.91	10.8	37	91	—	0.0
Mixture P.T. 2	5.–	0.587	0.052	0.85	9.6	135	83	1.07	–
Mixture P.T. 3	5.–	0.555	—	0.90	—	192	95	0.540	–
Mixture P.T. 4	5.4	0.556	0.035	0.97	15.4	116	85	0.560	–
Mixture P.T. 5	4.–	0.600	0.042	0.67	9.5	90	85	0.882	–

The following remarks are pertinent to this matter. The ultrafiltration residue mainly consists of a mixture of "specific" and "non-specific" proteins.

This residue is subsequently subjected to ammonium sulfate fractionation resulting in a separation of "specific" toxoid-protein and "non-specific" proteins. This operation moreover eliminates the non-proteinous matter that still remained in the ultrafiltration residue. So the total N content of the final product practically equals the protein nitrogen (P.N.). As the estimation of the total N content is much simpler than the protein-N determinations, the latter values have only been established as an exception.

As is apparent from the graph, the "specific" toxoid-protein is evidently salted-out prior to the accompanying other protein components that are unwished for. This implies that a rather high purification factor is already attained with only a single precipitation in the ultrafiltration residue at one specific ammonium sulfate

E 2.

tetanus toxoid.

ESIDUE			PRECIPITATED ULTRAFILTRATION RESIDUE							
Lf/ mg otal N	Lf/ mg P.N.	P.F. based on total N	% $(NH_4)_2SO_4$ used for precipitation	Lf/ ml	% of the ultr. residue recovered	% total N	% P.N.	Lf/ mg total N	Lf/ mg P.N.	P.F. based on total N of ultra filtrate
17.6	190	23.5	—	—	—	—	—	—	—	—
—	109	—	25.0	42	95	—	0.0115	—	365	—
12.6	—	10.4	18.0	90	92	0.293	0.164	30.6	55	2.4
35.5	—	39.4	27.0	145	85	0.181	—	80.0	—	2.3
20.7	—	21.3	19.0	116	85	0.232	—	50.0	—	2.4
10.2	—	15.3	21.0	155	100	0.700	—	22.1	—	2.2

concentration. This is in contrast with the fractionation of the diphtheria toxoid ultrafiltration residue.

From various tetanus toxoids thus purified a few batches of "Tetanus-Phosphate-Toxoid" (Tetanus-P. T.) were prepared, all having the following composition:

10 Lf/ml purified tetanus toxoid
10 mg/ml aluminium phosphate
Preservative: merthiolate 1 : 10.000.

In addition the preparation of mixed prophylactics with purified diphtheria toxoid and/or whooping cough-vaccine, of the under mentioned composition, has recently been taken up in our Institute:

Tetanus-Diphtheria-P.T.
10 Lf/ml purified tetanus toxoid
30 Lf/ml purified diphtheria toxoid
10 mg/ml aluminium phosphate;

Tetanus-Diphtheria-Whooping cough-P.T.

10 Lf/ml purified tetanus toxoid
30 Lf/ml purified diphtheria toxoid
40 × 10^9 whooping cough bacilli/ml
10 mg/ml aluminium phosphate
Preservative : merthiolate 1 : 10.000.

IMMUNIZATION EXPERIMENTS.

With the various tetanus prophylactics, groups of guinea-pigs, comprising 12 animals each, were immunized by methods and with results listed in table 3.

TABLE 3.

Immunization tests with different tetanus prophylactics.

Prophylactic	first injection ml	lapse in weeks	second injection ml	lapse in weeks	third injection ml	lapse in weeks	serum titre A.U./ ml
Fluid, crude Tetanus toxoid	1.0	3	1.0	3	1.0	3	2.—
Fluid, crude Tetanus toxoid	1.0	3	1.0	3	1.0	3	11.—
Tetanus-P.T.1	0.5	4	0.5	—	—	4	10.—
Tetanus-P.T. 2	0.5	4	0.5	—	—	4	33.—
Tetanus-P.T. 3	0.5	4	0.5	—	—	4	26.—
Tetanus-P.T. 4	0.5	4	0.5	—	—	4	22.—
Tetanus-P.T. 5	0.5	4	0.5	—	—	4	47.—
Tetanus-Diphtheria-P.T. 1	5	4	0.5	—	—	4	22.—
Tetanus-Diphtheria-Whooping cough P.T. 1	0.5	4	0.5	—	—	4	22.—

Concerning this it is pointed out that the crude fluid tetanus toxoid was injected subcutaneously whereas the adsorbed prophylactics were always administered by intramuscular injection.

Information gained from our experience with various diphtheria prophylactics indicates that the difference in the method of injection of the adsorbed tetanus toxoid (intramuscular) and the unadsorbed product (subcutaneous) cannot essentially affect the results of the tests.

The serum titres were determined in a pooled sample of the sera of all treated animals of one group.

From the results of these immunization tests it is evident that, with an exception of the Tetanus-P.T., all adsorbed tetanus prophylactics as well as the combinations with purified diphtheria toxoid and whooping cough vaccine, induce considerably higher serum titres and in a shorter lapse of time, than the unadsorbed tetanus toxoid does.

Summary.

1. Purification procedures are described in which crude tetanus toxoid is purified and concentrated, primarily by ultrafiltration and secondarily by fractionation with ammonium sulfate.

2. From the total protein-complex, present in the ultrafiltration residue, the tetanus antigen can be specifically salted out at a lower ammonium sulfate concentration than the accompanying ballast-proteins. This makes possible a sharp separation of this protein-complex, while good purification factors are obtained.

3. Of the tetanus toxoids thus purified, adsorbed prophylactics were prepared by adsorbing these products on aluminium phosphate, whether or not in combination with purified diphtheria toxoid or whooping cough vaccine.

4. With these adsorbed prophylactics, immunization tests on guinea-pigs were initiated. Purified and adsorbed tetanus toxoid as well as its combination with above mentioned diphtheria toxoid and whooping cough vaccine turned out to immunize much better than crude, unadsorbed tetanus toxoid.

Literature.

1. A. C. Brandwijk and A. Tasman, Ned. Tijdschr. Hyg. Microbiol. Ser. **8**, 94, 1933. – 2. A. C. Brandwijk and A. Tasman, Z. Immf. Exp. Ther. **78**, 540, 1933. – 3. J. F. H. de Graaf, A. Tasman and A. C. Brandwijk, Ned. Tijdschr. Geneesk. **94**, 2896, 1950. – 4. L. Levine and J. L. Stone, J. Immunol. **67**, 235, 1951. – 5. P. J. Moloney and J. A. Hennesy, J. Immunol. **48**, 345, 1944. – 6. A. L. Noordam, A. C. Brandwijk, A. Tasman and W. Aeg. Timmerman, Ned. Tijdschr. Geneesk. **93**, 4014, 1949. – 7. J. D. van Ramshorst, The preparation of diphtheria-prophylactics. Thesis, Utrecht 1951.

(Department of Bacteriology and Experimental Pathology of the Institute for Preventive Medicine, Leiden, Holland).

STUDIES ON IMMUNITY IN POLIOMYELITIS ¹)

II. ADAPTATION OF A STRAIN OF POLIOMYELITIS VIRUS ON MICE, AND IMMUNIZATION EXPERIMENTS WITH THE MOUSE-ADAPTED VIRUS ²)

by

J. D. VERLINDE, B. BEEM and **A. KLARENBEEK**

(Received July 8, 1952).

The AK strain of poliomyelitis virus was isolated in 1949 in this laboratory (18) from the stools of a girl, 2 years of age, who was suffering from poliomyelitis, on the 6th day after onset of paralysis. So far it has been carried through 21 passages either in rhesus or in cynomolgus monkeys, in which paralysis and characteristic anterior horn lesions developed regularly following intracerebral, and occasionally following intramuscular or intratonsillar inoculation. The incubation period varied from 4 to 27 days, with an average of 9 days.

Several unsuccessful attempts were made to produce paralysis in mice with monkey cord of various passages, but the 17th passage proved pathogenic for mice.

At that time we tried to confirm the observations of HABEL and LI (2) and LI and HABEL (7), who succeeded in producing paralysis in mice by intraspinal inoculation of the Lansing and the Leon strain. This technique was then applied to both these strains and to the 17th monkey passage of the AK strain.

The Leon strain produced paralysis in 5 out of 8 mice after an incubation period of 10 days. Two mice died on the 3rd and 5th day

---

¹) Part I: J. D. VERLINDE and A. KLARENBEEK, Arch. Virusforschung in print.

²) Aided by a grant from the Dr Simon Baruch Foundation.

without showing paralysis, and one survived. Cord suspension of the paralyzed mice produced paralysis in the rhesus monkey after an incubation period of 7 days.

The Lansing strain also produced paralysis in mice after an average incubation period of 14 days when inoculated intraspinally.

Surprising results were obtained with the 17th monkey passage of the AK strain. Paralysis developed in all of 6 mice 4 and 5 days following intraspinal inoculation.

Since hyaluronidase had proved to increase the paralytic attack rate in monkeys following intramuscular inoculation of the AK and the Aycock strain (12), groups of 8 mice were inoculated with a 10% monkey cord suspension of various strains of poliomyelitis virus (Brunhilde, Lansing, Y-SK, Leon and AK) either intracerebrally or intramuscularly, the latter with and without hyaluronidase. From the intramuscularly inoculated mice only those having received the AK strain yielded positive results in the series with hyaluronidase as well as in those without the enzyme preparation. The AK strain, however, proved also capable to produce paralysis in mice following intracerebral inoculation. The average incubation period was 3 days when inoculating intracerebrally, and 5 days when using the intramuscular route. Both brain and cord of the mice could readily be transferred serially to fresh mice, but it proved avirulent for rhesus and cynomolgus monkeys.

PROPERTIES OF THE MOUSE-ADAPTED AK STRAIN.

The intracerebral and intramuscular mouse infectivity titre of the 17th monkey passage was approximately 10^{-5}. The titre increased to 10^{-6} in the first, and to $10^{-7.5}$ and $10^{-8.5}$ in the second and third mouse passage. The latter level was maintained in subsequent passages.

When using potassium veronal buffer solution (JUNGEBLUT and HORVATH (5)) as diluent, the mouse passage virus agglutinated human 0 cells to a titre of 1/40 to 1/160. Monkey cord, however, failed to agglutinate red cells.

The development of paralysis in mice following intracerebral and intramuscular inoculation, the relatively short incubation period, the high mouse infectivity titre, and the haemagglutinating capacity are properties which remind of those of the viruses of the Columbia SK group. Our first thought was that of a laboratory contamination with one of these viruses, although animals infected with them were kept in other animal houses.

When looking at the dates, it appeared that Columbia SK virus had not been handled in the laboratory the month preceding the inoculation of mice with the AK strain. Two new members of this group of viruses, the F and the Ortlieb strain, which had been isolated in Germany [Bieling (1), Vivell and Mauer (20)] and studied in this laboratory (16), had not been handled on the same days as the AK strain. Moreover, mice and monkeys were kept in completely separated animal houses, and monkeys, when inoculated with different strains, were kept in different cages separated by a tiled brick wall.

Nevertheless, the mouse inoculations were repeated several times under very carefully controlled conditions. Mice inoculated with monkey cord of the 17th passage of the AK strain were placed in a cellar, in which animals had never been kept. In order to investigate the possibility of contamination in the animal departments, control mice inoculated with monkey cord of other strains, including the Brunhilde strain and the 10th, 11th, 12th, 13th, 15th and 16th passage of the AK strain were kept in an animal house in which mice were present, which had been inoculated with viruses of the Columbia SK group, without taking any precaution to prevent contamination. No paralysis occurred in the latter animals in repeated tests, so that the possibility of contamination in the animal house seems most unlikely. The animals which had been inoculated with the 17th passage of the AK strain, and which had been kept in the cellar, came down with paralysis regularly. Thus, there seems to be circumstantial evidence, that the mouse-pathogenic AK virus was present in the 17th monkey passage (rhesus).

A new monkey-line was made starting from the 16th passage. The next passages, which have been made as far as the 21st, were regularly pathogenic for monkeys, but the 19th passage proved also virulent for mice. The mouse virus derived from the 19th passage had the same properties as that which originated from the 17th passage, with the only difference, that it had maintained its monkey-pathogenicity.

Besides the properties which had the mouse AK line in common with the viruses of the Columbia SK virus, there were also differences.

The most striking difference between the viruses of the Columbia

SK group studied in this laboratory, and the mouse-adapted AK strain derived from the 17th passage, was the lack of monkey-pathogenicity of the latter.

It has been shown earlier (4, 13), that Columbia SK virus produces paralysis in cynomolgus monkeys, and that F and Ortlieb virus produce paralysis in both cynomolgus and rhesus monkeys (16). The mouse AK line derived from the 17th passage proved avirulent for 17 rhesus, 22 cynomolgus and 1 cercopithecus monkey following intracerebral inoculation of 1 ml of a 10% suspension of mouse brain, and for 16 cynomolgus monkeys following intramuscular inoculation of the same dose. However, paralysis occurred in one out of 3 monkeys, which had been inoculated intramuscularly, and which had been given one simultaneous injection with cortisone (Shwartzman and Fisher (10)).

Preceding experiments had shown (19) that Columbia SK virus can be grown in the chick-embryo following amniotic inoculation for more than 20 passages, whereas the F strain failed to grow in the chick-embryo, and the Ortlieb strain could be maintained for a few passages only (16). When the same technique was applied to the mouse AK strain, it appeared to behave in the chick-embryo like the Ortlieb strain, and different from Columbia SK and F virus (table 1).

Table 1.

Growth of AK mouse virus in the chick-embryo.

egg-passage	mouse infectivity titres in repeated tests			
	mouse-virus derived from the			
	17th monkey passage		19th monkey passage	
0	10^{-8}	10^{-8}	10^{-5}	10^{-5}
1	10^{-4}	10^{-1}	10^{-1}	—
2	10^{-5}	10^{-3}	10^{-4}	—
3	10^{-4}	—	10^{-1}	
4	10^{-2}	—	10^{-2}	
5	—	—	10^{-2}	
6	—	—	—	
7	—	—	—	

TYPING BY CROSS-IMMUNITY EXPERIMENTS.

Twenty-four monkeys were immunized by three intramuscular inoculations of 1 ml of pooled monkey cord of the 12th and 14th passage of the AK strain. The cord suspension had been adsorbed to aluminium phosphate and was attenuated by ultraviolet irradiation according to a method which has been described previously (17). Twenty-four monkeys were immunized in the same way with F virus. Six monkeys which had survived a non-paralytic attack following intracerebral inoculation of the Aycock strain were also included. The animals were challenged either intramuscularly by Columbia SK virus, or intracerebrally by one of the following strains: Brunhilde, Aycock, Leon, AK monkey-line, F mouse-line, the challenge dose being 100 PD_{50}.

Table 2.

Cross-immunity experiments.

Number of monkeys	Preceding immunization	Challenge virus	Route of inoculation	Paralyzed animals	
				immunized	controls
5 cynomolgus	AK monkey-line	Col. SK	i.m.	0/5	4/5
3 rhesus		Brunhilde	i.c.	1/3	3/3
3 cynomolgus		Aycock	i.c.	2/3	3/3
3 rhesus		Leon	i.c.	3/3	3/3
10 rhesus		AK (monkey)	i.c.	2/10	4/4
8 rhesus	F mouse virus	F	i.c.	2/8	4/4
4 rhesus		Brunhilde	i.c.	3/4	4/4
4 rhesus		Aycock	i.c.	2/4	4/4
4 rhesus		Leon	i.c.	4/4	4/4
4 rhesus		AK (monkey)	i.c.	2/4	4/4
1 rhesus	Aycock (non-paralyzed survivors)	Brunhilde	i.c.	0/1	2/2
2 rhesus		Leon	i.c.	2/2	2/2
3 rhesus		AK (monkey)	i.c.	0/3	3/3

Table 2 shows, that the AK monkey-line has produced resistance to intracerebral challenge in 80% of the monkeys against homologous virus. When this fact is taken into consideration, the AK strain would appear to be related to the Brunhilde type, to the

Columbia SK group, and, perhaps, to the Aycock strain, which has been shown to be related to the Lansing type (11). Monkeys immunized with the F strain showed a partial resistance to homologous virus, to the Lansing type and the AK strain, and, perhaps, a little to the Brunhilde type. Finally, the non-paralyzed surviving monkeys following inoculation of the Aycock strain appeared to be resistant to the AK strain, and one animal to the Brunhilde type.

Thus, the cross-immunity experiments are suggestive for a partial immunologic relationship of the monkey-line of the AK strain with the Columbia SK group, the Lansing type, and the Brunhilde type.

TYPING BY NEUTRALIZATION EXPERIMENTS.

Since it was shown, that the AK mouse-line as well as the viruses of the Columbia SK group, including the F and the Ortlieb strains, are capable to produce paralysis not only following intracerebral and intraperitoneal, but also following intramuscular inoculation of mice, neutralization tests were carried out by intramuscular inoculation of virus-serum-mixtures.

The mice used in these experiments were white, black-eyed hybrids of Rhodesfarm and Extreme Dilute mice, bred in the Central Breeding Station of the National Health Research Council. These animals proved highly susceptible for neurotropic viruses, and the titrations with the viruses of the Columbia SK group, and the mouse-line of the AK strain were carried out by intramuscular inoculation of 0.05 ml of virus-serum-mixtures. Titrations with the Lansing strain were carried out by intracerebral inoculation.

Immune sera were prepared in rhesus monkeys by 2 to 3 intramuscular injections of either monkey cord, or mouse brain and cord, with Bayol F and Arlacel A according to the method described by SALK, LEWIS, YOUNGNER and BENNETT (9).

Tenfold dilutions of virus were mixed with equal volumes of undiluted immune serum. Normal monkey serum was used in the control series. The mixtures were placed at room temperature for 2 hours, and then inoculated into groups of 4 mice.

Table 3 shows, that the AK mouse-line is neutralized completely by homologous immune serum and by F immune serum, and to a lesser degree by immune serum against the AK monkey-line and by Columbia SK serum. The difference in the degree of neutralizing

Table 3.

Neutralization tests.

Immune serum against	Average log of neutralization index as determined in 2 to 3 experiments Virus AK mouse-line	 Lansing strain	 F strain
AK monkey-line	3.3	>2	3.8
AK mouse-line	5.3	>2	5.0
F mouse virus	5.0	1.5	6.0
Columbia SK virus	2.5	—	6.0
Lansing	—	>2	—
Aycock	—	>2	—
Brunhilde	—	—	—
Leon	—	—	—
Theiler FA	—	—	—
Theiler GD VII	—	—	—

potency between AK monkey-antiserum and AK mouse-antiserum may be due to a difference in antigenicity of the monkey-line and the mouse-line.

The neutralization indices with F virus are approximately as high as those with AK mouse virus, with the exception, that the latter is only partly, and the former completely, neutralized by Columbia SK immune serum. Thus, AK virus seems to be serologically more closely related to F virus than to Columbia SK virus. The Lansing strain is neutralized not only by homologous sera (anti-Lansing and anti-Aycock), but also by AK immune serum and less by F immune serum. This shows a serologic relationship between AK virus and the Lansing type, and also between F virus and the Lansing type. AK-virus, however, seems to be more closely related to the Lansing type than F virus.

No serologic relationship could be shown between AK virus and the Brunhilde-, Leon- and Theiler FA and GD VII viruses.

These neutralization experiments correspond so far with the cross-immunity tests, that the AK strain seems to be related with both the Lansing type and the Columbia SK group. Although a certain relationship with the Brunhilde type was shown by cross-immunity experiments, no evidence of a serological relationship with this type was obtained.

Since the mouse-line of the AK strain proved capable of agglutinating human 0 cells, the same immune sera were tested for their haemagglutination-inhibiting properties. When using 8 haemagglutinating units of virulent mouse brain suspension, the haemagglutination by AK mouse virus was inhibited both by homologous immune serum, and by immune sera against the viruses of the Columbia SK group to a titre of 1/640 to 1/1280. The other sera mentioned in table 3 failed to inhibit the haemagglutination.

IMMUNIZATION EXPERIMENTS.

Since the AK mouse-line derived from the 17th monkey passage proved practically avirulent for monkeys, and the cross -immunity experiments suggested an immunizing pattern partially covering the Columbia SK group, the Lansing type and the Brunhilde type, this strain could be important as a living immunizing agent against two types of poliomyelitis virus and against infections with similar intermediate strains like F and Ortlieb virus. The latter viruses have presumably been isolated from human beings suffering from a poliomyelitis-like disease, which has already been called parapoliomyelitis (VIVELL and MAUER (20)).

Two immunization experiments have been carried out so far. In the first experiment 17 rhesus monkeys, 1 cercopithecus and 1 cynomolgus were inoculated intracerebrally with 0.5 ml of a 10% brain suspension of the first and 4th mouse passage of the AK strain. None of the animals showed signs of illness during the period

Table 4.

Immunity following intracerebral inoculation of the mouse-adapted AK strain.

Monkeys	Intracerebral challenge 47 days later		controls paralyzed
	virus	paralyzed	
1 cercopithecus	AK 18th monkey passage	1/1	
1 cynomolgus	AK 18th monkey passage	1/1	
5 rhesus	AK 18th monkey passage	2/5	3/3
3 rhesus	Brunhilde	3/3	3/3
3 rhesus	Aycock	1/3	3/3
3 rhesus	Leon	3/3	3/3
3 rhesus	F	1/3	3/3

of observation. They were reinoculated intracerebrally 47 days later with 100 PD_{50} of various strains. The results are listed in table 4, which shows, that the AK mouse virus produces a certain degree of resistance to a subsequent intracerebral reinoculation with the AK monkey-line, Aycock virus and F virus. There was no evidence of resistance to the Brunhilde and the Leon type.

The intracerebral challenge is a heavy immunity test, and there seems to be little reason to aim at a solid immunity of the central nervous system. It is generally agreed, that only a small proportion of the persons infected with poliomyelitis virus develop symptoms of infantile paralysis, whereas the vast majority of poliomyelitis infections takes the course of minor illness or that of a subclinical infection. Haematogenous spread in experimentally infected monkeys has been shown for the viruses of the Columbia SK group (VERLINDE and HOFMAN (16)) for the Brunhilde and the Lansing types (HORSTMANN (3)), and for the Leon type (VERLINDE and BEEM (14)), whereas the latter authors also presented circumstantial evidence for a secondary neural spread of the virus from muscle tissue. Hence, a specific prevention of poliomyelitis has not to be directed primarily to immunity of the central nervous system, but to peripheral immunity (portal of entry, muscle tissue, blood).

Since a test for peripheral immunity, e.g. following intramuscular inoculation would require large numbers of monkeys, a method was chosen, by which paralysis following peripheral inoculation would occur in a high percentage of the monkeys. SHWARTZMAN and FISHER (10) showed, that the susceptibility of hamsters for poliomyelitis virus is markedly increased by cortisone, and this method was also applied in our monkey experiments.

Four groups of 2 rhesus monkeys received one single intramuscular injection of 25 mg of cortisone acetate, which was repeated one week later. [1]) The animals were inoculated simultaneously with the first injection of cortisone with 1 ml of a 10% suspension of monkey cord, each group receiving either the AK strain, Brunhilde, Lansing (Aycock strain) or Leon type. In all but one of the animals paralysis developed after an incubation period ranging from 6 to 26 days. Typical anterior horn lesions were found in the paralyzed animals.

Then a second immunization experiment was carried out, in which rhesus monkeys were fed mouse-adapted AK virus. MELNICK and LEDINKO (8) had shown earlier, that immunity could be produced in cynomolgus monkeys following feeding Y-SK virus. This strain, however, was not avirulent, but produced paralysis in a certain percentage of the animals.

---

[1]) The cortisone was kindly supplied by N.V. Organon, Oss, Holland.

In our second experiment twelve rhesus monkeys were fed second mouse passage virus of the AK strain. Two or three feedings with an interval of 4 to 7 days were given, the dose in each feeding being a 10% suspension of one virulent mouse brain, i.e. approximately 3 ml. The virus was introduced into the mouth by a pipette. The stools of the monkeys were tested for the excretion of the virus by intraperitoneal inoculation of mice. Virus was recovered from the stools from the 5th to the 8th day following feeding.

Pooled serum taken just prior to feeding and 47 days later was examined for the presence of neutralizing antibodies against the mouse-adapted AK strain. It appeared from this experiment, that the first serum did not neutralize the virus, whereas the second serum had a weak neutralizing capacity (2 logs).

The animals were challenged 47 days after the first feeding by intramuscular inoculation of 500 intracerebral PD_{50} doses of the types of poliomyelitis virus. A simultaneous intramuscular injection with 25 mg of cortisone acetate was administered, which was repeated one week later. Since the Leon type produced paralysis in almost 100% of the animals following intramuscular inoculation without cortisone, this drug was not given to monkeys which were challenged with the Leon strain.

Table 5.

Immunity following oral administration of the mouse-adapted AK strain.

Monkeys	Intramuscular challenge and cortisone treatment 47 days later		
	virus	paralyzed	controls paralyzed
3 rhesus	AK 18th monkey passage	0/3	2/2
3 rhesus	Brunhilde	1/3	2/2
3 rhesus	Aycock	0/3	1/2
3 rhesus	Leon	3/3	2/2

All but one of the control animals listed in table 5 came down with paralysis following intramuscular inoculation of poliomyelitis virus and cortisone, whereas only one out of 9 immunized monkeys, developed paralysis following peripheral challenge with AK, Brunhilde, and Aycock virus. Although the number of animals is too small to draw definite conclusions, the results are highly suggestive for the production of peripheral resistance in animals immunized with the mouse-adapted AK strain by oral administration.

DISCUSSION.

The AK strain isolated from a human case of paralytic poliomyelitis presents all the characteristics of poliomyelitis virus. A mouse pathogenic mutant (?) was derived from the 17th monkey passage, which proved practically avirulent for monkeys. Serologically, both the simian and the mouse AK strain appeared to be related both to the Lansing type and to the Columbia SK group of viruses. On an immunological basis, the strain appeared to be related also to the Brunhilde type. Although the mouse-adapted strain has a high infectivity titre for mice, and is able to agglutinate human 0 cells, there are differences with other members of the Columbia SK group. The mouse-adaptation could frequently be repeated under such carefully controlled conditions, that a laboratory contamination with one of these virus can be excluded.

It is a matter of speculation, whether the mouse-adapted strain has to be called a mutant of the simian strain, or whether it is a mixed virus (Lansing type and Columbia SK group), which was already present in the original human material, or an intermediate strain. No evidence has been obtained, that carriers of a virus of the Columbia SK group have occurred among our monkeys. Several serum samples of fresh animals have been examined for the presence of neutralizing antibodies against the Columbia SK group, but they were all negative. If such carriers had occurred, one would expect that other types of poliomyelitis virus, which are regularly transferred in this laboratory, would have presented also a suddenly appearing mouse infectivity as happened in the 17th and 19th passage of the AK strain.

It can be provisionally concluded from small-scale immunization experiments, that the mouse-adapted AK strain produces a certain degree of immunity against homologous virus, as well as against the Lansing and the Brunhilde type and against the Columbia SK group. Resistance to the Lansing type and the Columbia SK group could be shown already when the monkeys were challenged by intracerebral route. By peripheral challenge, and simultaneous injection of cortisone, also a resistance to the Brunhilde type was shown.

The results obtained so far, are highly similar to those obtained by KOPROWSKI, JERVIS and NORTON (6) in their experiments with the TN strain, which, however, did not appear fully avirulent. Both the AK and the TN strain produced antibodies against the homologous type, but a certain degree of resistance to heterologous virus, with the exception of the Leon type, was produced in immunity experiments.

If the AK strain might appear to be a suitable immunizing strain in humans, the immunological relationship not only with the Lansing and the Brunhilde type, but also with the Columbia SK group of viruses seems to be advantageous, at least for Europe, since both the Lansing and the Brunhilde type have been found in the Netherlands (15), whereas strains of the Columbia SK group have been isolated from cases of "parapoliomyelitis" in Germany.

Summary.

The AK strain of poliomyelitis virus was shown to be pathogenic for mice in its 17th monkey passage. The strain was serologically related both to the Lansing type and to the Columbia SK group of viruses. It differed in pathogenicity from the members of the Columbia SK group, except in its high mouse infectivity and haemagglutinating capacity, so that a laboratory contamination with one of these viruses could be excluded. Moreover, the adaptation was frequently repeated under carefully controlled conditions. The mouse-adapted AK strain proved practically avirulent for rhesus and cynomolgus monkeys, but it produced a certain degree of resistance of the central nervous system to the Lansing type and to the Columbia SK group. When rhesus monkeys were fed mouse AK virus, the virus was excreted in the stools, antibodies against the homologous virus developed, and the animals proved resistant to peripheral challenge with the Lansing type, the Columbia SK group and with the Brunhilde type. Peripheral challenge was possible, since it was shown, that paralysis developed in intramuscularly inoculated monkeys with the various types of poliomyelitis

virus, and simultaneous injection of cortisone. The origin of the virus and the possibility of its use as immunizing agent in man are discussed.

References.

1. R. Bieling, Wien. med. Woch. **102**, 106, 1952. – 2. K. Habel and C. P. Li, Proc. Soc. exp. Biol. a. Med. **76**, 357, 1951. – 3. D. M. Horstmann, Proc. Soc. exp. Biol. a. Med **79**, 417, 1952. – 4. C. W. Jungeblut, Proc. Soc. exp. Biol. a. Med. **72**, 532, 1949. – 5. C. W. Jungeblut and B. Horvath, Proc. Soc. exp. Biol. a. Med. **77**, 672, 1951. – 6. H. Koprowski, G. A. Jervis and Th. W. Norton, Amer. Journ. Hyg. **55**, 108, 1952. – 7. C. P. Li and K. Habel, Proc. Soc. exp. Biol. a. Med. **78**, 233, 1951. – 8. J. L. Melnick and N. Ledinko, J. Immun. **67**, 213, 1951. – 9. J. E. Salk, L. J. Lewis, J. S. Youngner and B. L. Bennett, Amer. J. Hyg. **54**, 157, 1951. – 10. G. Shwartzman and A. Fisher, J. exp. Med. **95**, 347, 1952. — 11. J. D. Trask, J. R. Paul and A. J. Vignec, Proc. Soc. exp. Biol. a. Med. **41**, 241, 1939. – 12. J. D. Verlinde, Arch. Virusforsch. **4**, 456, 1952. – 13. J. D. Verlinde, P. de Baan and J. A. Vercruysse, Antonie van Leeuwenhoek **16**, 9, 1950. – 14. J. D. Verlinde and B. Beem, Antonie van Leeuwenhoek **18**, 251, 1952. – 15. J. D. Verlinde and B. Beem, unpublished data. – 16. J. D. Verlinde and B. Hofman, Arch. Virusforsch. in print. – 17.J. D. Verlinde and A. Klarenbeek, Arch Virusforsch. in print. – 18. J. D. Verlinde en E. Nihoul, Ned. Tijdschr. v. Geneesk. **94**, 1186, 1950. – 19. J. D. Verlinde, P. Waller-Fetter and P. de Baan, Antonie van Leeuwenhoek **17**, 183, 1951. – 20. O. Vivell und R. Mauer, Z. Imm. forsch. **104**, 246, 1952.